STUDIES ON THE DEGRADATION OF AFLATOXINS BY AMMONIATION

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DEDICATION

For Colette.

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ABSTRACT

Chapter (1) defines mycotoxins and describes some of the more important members of this class of compounds. The need for effective chemical treatments to detoxify aflatoxin-contaminated commodities is discussed, and the literature is then reviewed with regard to acidic, oxidative, bisulphite, formaldehyde, and alkaline detoxification procedures.

In Chapter (2), initial studies on the treatment of aflatoxin B_1 with ammonium hydroxide are described. Aflatoxin D_1 and MW206 were isolated as products of the reaction, and synthesis of these two compounds was undertaken. Two synthetic approaches to MW206 are described, the second being successful. Model compounds related to aflatoxin D_1 were prepared, but the methodology was not compatible with the synthesis of aflatoxin D_1 itself.

Chapter (3) describes how the ammoniation of aflatoxin B_1 can be studied by nmr methods. Deuterated ammonium hydroxide was used to follow the reaction *in situ*. Comparison of products resulting from varying ammoniation times by nmr also allowed the progress of the reaction to be monitored.

Chapter (4) describes the ammoniation of aflatoxin G_1. MW206, aflatoxin GD_1, lactone ring-opened aflatoxin GD_1, and parasiticol were isolated from the reaction mixture. Model compounds related to parasiticol and aflatoxin GD_1 were synthesised, and these aided identification of

parasiticol and aflatoxin GD_1 .

ABBREVIATIONS

Ac	-	Acetyl
Bz	-	Benzyl
DMF	-	N,N-Dimethylformamide
Me	-	Methyl
MW206	-	Molecular weight 206 compound
nmr	-	Nuclear magnetic resonance
nOe	-	Nuclear Overhauser effect
THF	-	Tetrahydrofuran

PUBLICATIONS

A. D. Pemberton and T. J. Simpson, in "Mycotoxins and Animal Feedingstuffs: Natural Occurrence, Toxicity and Control", J. E. Smith, Ed., C.R.C. Uniscience, in press.

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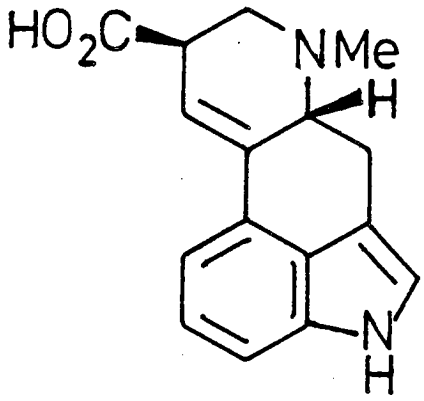
CHAPTER 1

INTRODUCTION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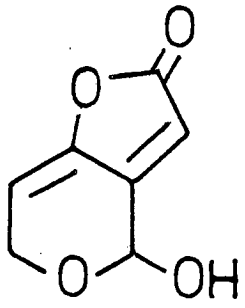
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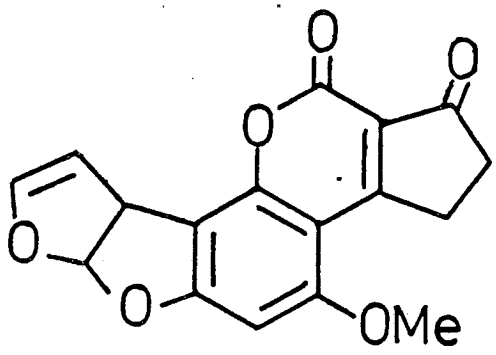
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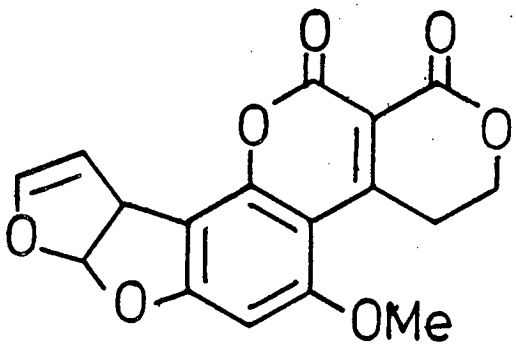
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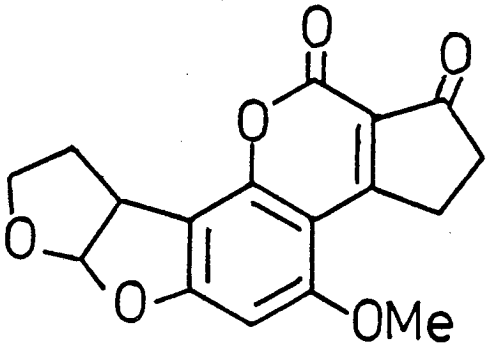
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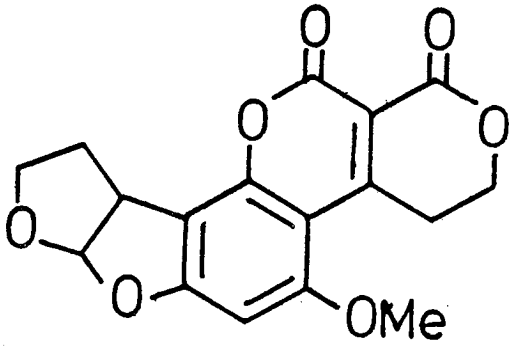
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1.1 MYCOTOXINS.

A mycotoxin is a toxic metabolite produced by a mould. Consequently, a mycotoxicosis is an illness caused by the ingestion of a mycotoxin, whereas a mycosis is due to invasion by the mould itself.

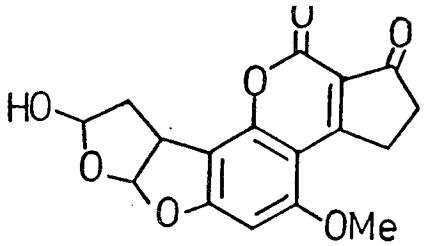
Incidents of the illness known as St. Anthony's fire, or ergotism, caused by the consumption of mouldy rye, have been periodically documented in Europe since the Middle Ages.[1] The appearance of the poisonous ergot grains on rye, consequent to its invasion by Claviceps purpurea, has been established as the cause of this disease; ergot consisting of a series of related alkaloids,[2] which are all derivatives of lysergic acid (1).

Although these facts were well established by the first half of this century, moulds were not generally regarded as being potentially harmful, but rather, in the light of the discovery of penicillin, may have been seen as being positively benificial. Indeed, patulin (2), isolated from Penicillium patulum, which was originally hailed[3] as a possible cure for the common cold, is now classed as a mycotoxin, and has been implicated in cases of toxicosis[4] affecting sheep and cattle.

1.1.1 Aflatoxins.

It was the advent of the aflatoxins that caused a change in the perceived threat from mycotoxins, and opened up a new area of research.

In 1960, numerous English farmers experienced severe losses of turkey poults due to a previously unknown



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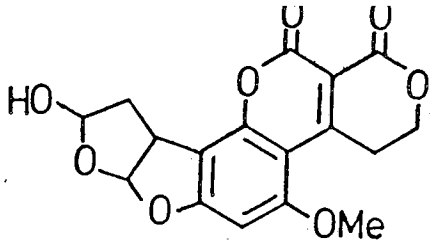

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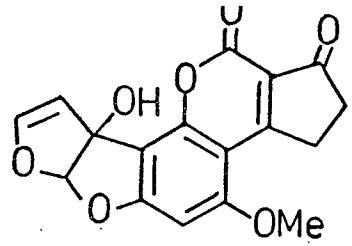

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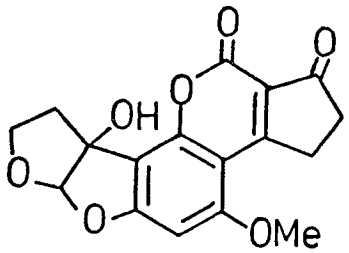

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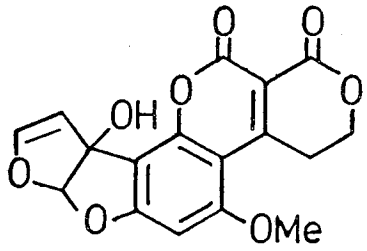

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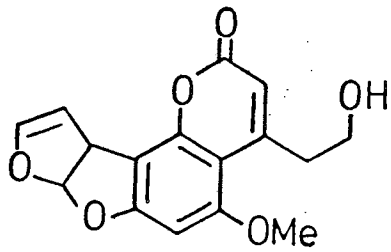

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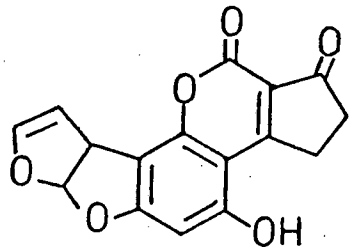

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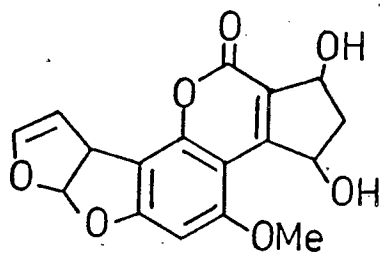

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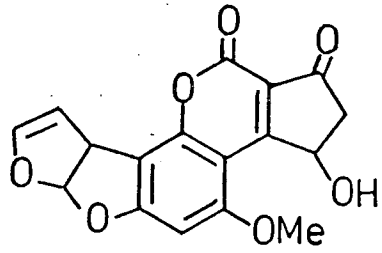

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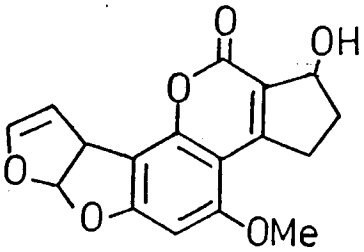

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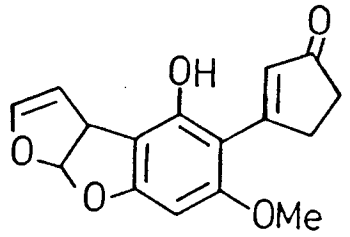
Figure (1)



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disease. The ensuing investigation[5] revealed a common link in that the proprietary "Brand X" feed was used in each case. The groundnut meal component of that feed, of Brazilian origin, was found to be mouldy, and sterile cultures of Aspergillus flavus spores obtained from the contaminated meal were shown to be toxic.[6] Thin layer chromatography (tlc) of crude A. flavus extracts showed two highly fluorescent spots, one blue, and the other green, when visualised under ultra-violet light. The compounds corresponding to these spots were isolated and characterised by Buchi et al.,[7] and named aflatoxin B_1 (3) and aflatoxin G_1 (4) respectively. Further characterisation of the extract[7] revealed the presence of the dihydro derivatives, aflatoxin B_2 (5) and aflatoxin G_2 (6). These four aflatoxins were all discovered to be acutely toxic, and in particular, aflatoxin B_1 was found to be the most potent hepatocarcinogen yet known in the rat.[8]

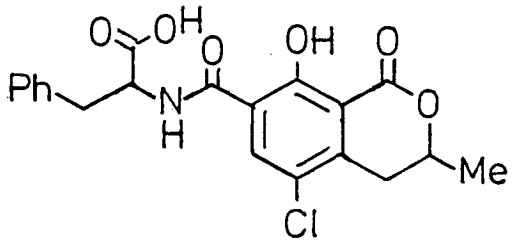
The alarming fact that certain peanut meals could cause cancer was compounded by the discovery of aflatoxins in some North American cottonseed and peanut meals,[9] resulting in the development of sensitive analytical techniques and the setting up of the 20 p.p.b. limit by the U.S. Food and Drug Administration (FDA).[10]

In the two decades following the discovery of aflatoxin B_1, many related compounds have been characterised, as shown in figure (1).

Aflatoxins B_{2a} (7) and G_{2a} (8) are hemiacetals derived by acid-catalysed hydration of aflatoxins B_1 and G_1 respec-

tively.[11] Aflatoxin M_1 (9) is the benzylic hydroxyl derivative of aflatoxin B_1, and is important as the major metabolite of B_1 found in milk.[12] Biological tests have established the acute toxicity[12] and carcinogenicity[13] of this compound, which has also been isolated from sheep urine[12] and from A. flavus cultures.[14] The dihydro derivative of aflatoxin M_1, i.e. aflatoxin M_2 (10), has also been characterised,[12] as has aflatoxin GM_1 (11),[15] the G_1 analogue of aflatoxin M_1. Another blue fluorescent metabolite, named aflatoxin B_3 (12) or parasiticol, has been isolated[15] in small quantities from A. flavus cultures, but on characterisation, it was seen to be more likely derived from aflatoxin G_1 (4) via a decarboxylation mechanism. Aflatoxin P_1 (13) is a phenolic metabolite of aflatoxin B_1 found[16] in the urine of rhesus monkeys, and two other metabolites have been isolated, following in vitro incubation of aflatoxin B_1 with monkey or human liver, namely aflatoxin H_1 (14)[17] and aflatoxin Q_1 (15).[18] Rhizopus species have also been shown to degrade aflatoxin B_1[19] to the reduced derivative, known as aflatoxin R_0 (16) or aflatoxicol. Completing the list is aflatoxin D_1 (17), obtained as a decomposition product from the reaction of aflatoxin B_1 with ammonium hydroxide.[20] This compound will be discussed in more detail later.

The intensification of research following the discovery of the aflatoxins has resulted in the identification of many more mycotoxins, although not all have economic significance comparable with the aflatoxins.



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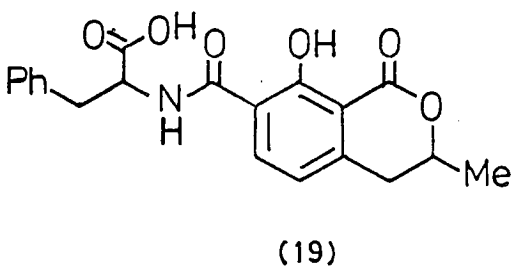

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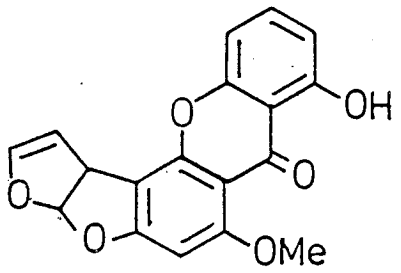

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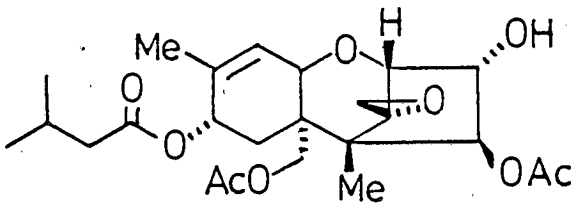

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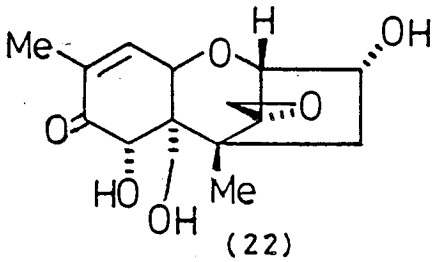

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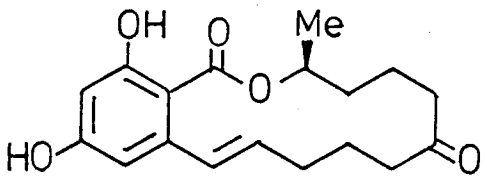


(23)

Figure (2)

Some of the more important of these mycotoxins, discussed below, are shown in figure (2).

1.1.2 Ochratoxins.

The ochratoxins were discovered[21] during a South African screening programme for toxigenic fungi. Ochratoxin A (18), produced by Aspergillus ochraceus and Penicillium viridicatum, is the most toxic of the ochratoxins;[22] the dechloro compound, ochratoxin B (19), being much less harmful. Cases of swine nephropathy seen in Denmark have been associated with the occurence of ochratoxins in mouldy barley,[23] but drastic reductions in toxin levels are observed when using such barley in alcoholic fermentation.[24] Roasting of ochratoxin-contaminated coffee beans causes a similar reduction.[25]

1.1.3 Patulin.

Another mycotoxin of economic significance, as mentioned earlier, is patulin (2). Since it is relatively rapidly degraded in many feedstuffs,[26] this toxin has been implicated in toxicoses by the isolation of patulin-producing strains of Penicillium patulum and Aspergillus clavatus from the affected feed.[4] Patulin in apple juice, produced by the Penicillium expansum rot of apples, has however been found to be quite stable,[27] but is removed on using the juice for alcoholic fermentation.[28]

1.1.4 Sterigmatocystin.

Sterigmatocystin (20) is a toxic and carcinogenic metabolite of Aspergillus versicolor.[29] Although not as toxic as aflatoxin B_1, with which it shares the dihydro-

furobenzofuran system, this compound is important as a precursor in the biosynthesis of aflatoxin B_1 by Aspergillus parasiticus.[30]

1.1.5 Trichothecenes.

The trichothecenes comprise a wide range of related metabolites, produced by a number of Trichothecium and Fusarium species. Human illness and mortality in Russia from the disease alimentary toxic aleukia (ATA), is linked[31] with the ingestion of cereal grains spoiled by trichothecene-producing Fusarium species. It has been suggested[31] that T-2 toxin (21) is the main toxin involved in ATA, and this compound has certainly been isolated from the feed of cattle suffering mouldy corn toxicosis.[32] Deoxynivalenol (DON) (22), also known as vomitoxin, is another trichothecene of economic significance since, although it is much less toxic than T-2 toxin, it causes vomiting and feed refusal in swine.[33] This toxin caused widespread spoilage of Canadian wheat in 1980,[34] being present in levels of up to 8.5 ppm.

1.1.6 Zearalenone.

Yet another Fusarium toxin is zearalenone (23), produced by Fusarium roseum at low temperatures, on corn and barley.[35] Animal consumption of zearalenone-contaminated feeds has been shown to cause hyperestrogenism,[36] particularly in swine.

1.2 DECONTAMINATION METHODS.

The evidence for the potential harmful effects of mycotoxins is now indisputible, so action must be taken to

eliminate such substances from the diet of man and domestic animals.

While it is sensible to store foodstuffs under conditions of temperature and humidity that minimise fungal growth, it is often the case that the product has been spoiled before harvest, and already contains considerable amounts of mycotoxins. The detection of mycotoxins is therefore very important, and routine screening methods for the detection of aflatoxins, ochratoxin A, patulin and sterigmatocystin are in operation.[1]

Consequently, once a product has been identified as being contaminated beyond a level fit for human or animal consumption, the problem arises of what to do with it. The removal of mouldy peanuts for example, by electronic sorting methods, is an attractive idea, but the fact that aflatoxins diffuse away from the mycelium means that residual contamination will remain.

Alternatively, aflatoxin-contaminated peanuts may be diverted to the production of edible oil, since the process results in an aflatoxin-free product,[37] but the toxins remain in the residual meal, which is often still unfit for use as food.

Reductions in mycotoxin levels are usually achieved during cooking processes, such as the dry roasting of peanuts, or the popping of corn. However, in the case of aflatoxins, the reduction is modest, and the consumer is unlikely to accept a product that has been contaminated at such a late stage.

The producer of a highly contaminated commodity may then be faced with the problem of its disposal, unless some treatment can be employed to reduce the toxic content to an acceptable level.

1.3 CHEMICAL TREATMENTS.

It should be noted that any chemical treatment of a contaminated feed must not only reduce the level of the contaminants, but also avoid the generation of any harmful by-products, in addition to retaining the effective food value of the product. While investigating such a detoxification process, attention should be paid to evaluating the risk from any by-products that can be identified, as well as to the extensive feeding trials required to determine the safety and acceptability of the end product.

The types of chemical processes used to detoxify aflatoxin-contaminated meals can be roughly grouped into five categories: acidic, oxidative, bisulphite, formaldehyde, and alkaline treatments. It is the latter, and in particular ammoniation, that has received the most attention in the literature.

1.3.1 Acidic Treatment.

An obvious target moiety for detoxification in the aflatoxin B_1 molecule is the vinyl ether double bond. Catalytic hydration of this double bond using trifluoroacetic acid[38] or cold dilute aqueous mineral acid[11] has been shown to produce the hemiacetal, aflatoxin B_{2a} (7). Similarly, aflatoxin G_1 reacts to give aflatoxin G_{2a}

(8). These compounds had previously been isolated as polar fluorescent metabolites of A. flavus,[11] and aflatoxin B_{2a} was synthesised[38] by Buchi as an intermediate in his early aflatoxin B_1 synthesis.

Since the toxicity of aflatoxin B_{2a} is only of the order of 1/200th that of aflatoxin B_1, there has been some effort to research the efficacy of acidic treatments in detoxifying aflatoxin-contaminated meals.

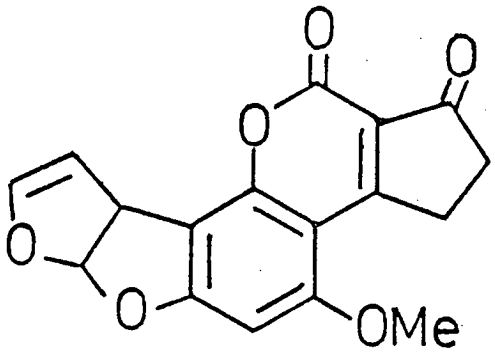
With a view to detoxification, Ciegler and Peterson reacted pure aflatoxin B_1 with aqueous citric acid, isolating aflatoxin B_{2a} as the major product.[39] The decrease in pH during the ensilage of corn also prompted an investigation[40] into the possible inactivation of aflatoxins in this acidic environment. Although a 96% reduction in aflatoxin B_1 levels was found during storage of contaminated shelled corn with 1.0 M HCl for 21 days, there was little or no decrease in toxin content during ensilage.

The kinetics of the acid-catalysed hydration have been studied, and a first order dependence of H^+ ion concentration was found.[41] From the results, the authors suggested acidification of alkaline soapstocks obtained from the processing of aflatoxin-contaminated oilseeds, at typically pH 1 and 100 °C for 10 minutes.

However, the resistance of the dihydro aflatoxins (B_2 and G_2) to acidic treatment makes it unlikely that such processes will reduce the aflatoxin content sufficiently.

1.3.2 Oxidative Treatment.

Aflatoxins are susceptible to oxidising agents; indeed



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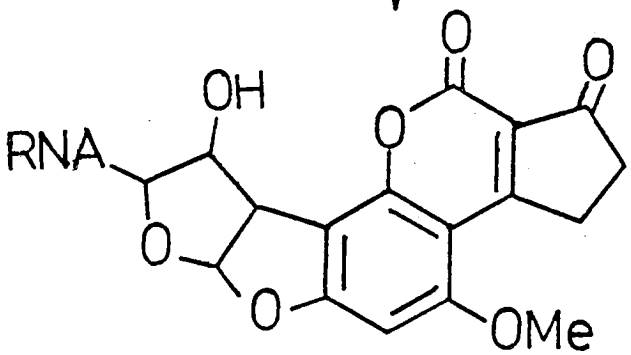
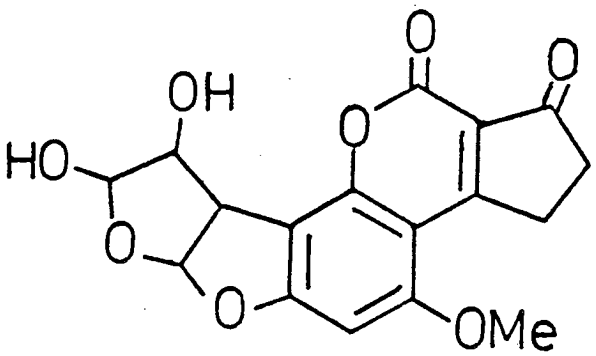
RNA | liver microsomes



H^+



(24)

Scheme (1)

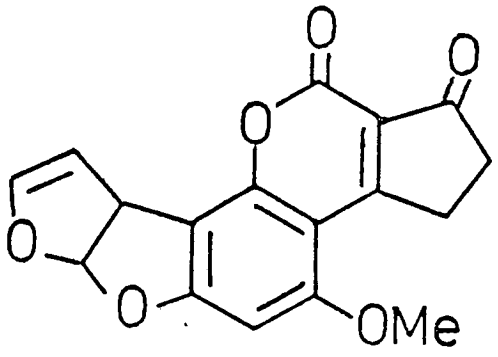
it is the ready epoxidation of aflatoxin B_1 by oxygenating liver enzymes that has been implicated in its extreme carcinogenicity,[42] resulting in a highly reactive epoxide intermediate, which binds to DNA.

The *in vitro* binding of aflatoxin B_1 to RNA in the presence of liver microsomes has been studied by Swenson;[43] acid hydrolysis of the complex releasing the dihydroxy derivative (24), as shown in scheme (1). This same compound was prepared by oxidation of aflatoxin B_1 with osmium tetroxide.[43] Hence, oxidation merits investigation as a detoxification process.

Sodium hypochlorite is used routinely, and is the reagent of choice in the laboratory, for decontamination of aflatoxin-contaminated apparatus. This has not been much investigated as a means of detoxifying contaminated foods and feeds, although Cater *et al*. found a reduction in aflatoxin content from 1000 ppb to non-detectable levels when employing sodium hypochlorite in an aqueous extraction process used on coconuts and peanuts.[44] The use of hydrogen peroxide in the same process gave similar results.

Alkaline hydrogen peroxide has been used to good effect in the removal of aflatoxin from peanut meal.[45] 97% Destruction was achieved on treatment of peanut meal containing 90 ppm toxins with 6% hydrogen peroxide at pH 9.5 for 30 minutes. The treated meal was found to be non-toxic to ducklings, and the protein efficiency ratio (PER) was not reduced.

The mechanism of this process probably entails initial



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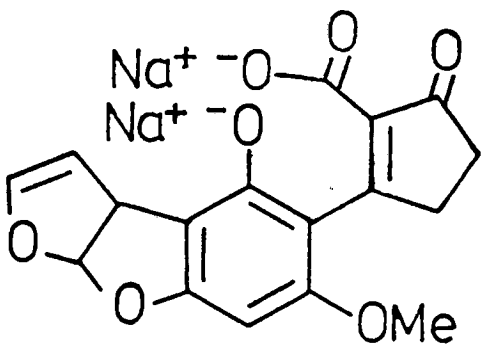


oxidation of
phenolic ring

Scheme (2)

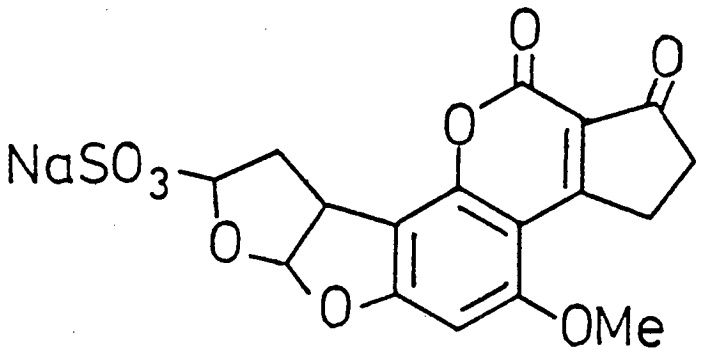
opening of the lactone ring in the alkaline conditions, followed by oxidation of the sensitive phenol so formed [scheme (2)]. Phenols are known to be susceptible to oxidative breakdown under these conditions.[46]

A different oxidation technique, ozonisation, has also been applied to some effect. Treatment of aflatoxin-contaminated peanut meal[47] at 30% moisture and 100 °C, with 25 mg min^{-1} of ozone gave a meal with greatly reduced toxin content, as evidenced by tlc and feeding experiments with ducklings and rats.

Further investigation into the ozonisation process was carried out in cottonseed and peanut meals.[48] Heating contaminated cottonseed meal at 22% moisture, 100 °C for 2 hours gave complete elimination of aflatoxin B_1. A similar effect was seen in peanut meal. Heating the meals in the presence of air gave a lesser reduction in aflatoxin B_1 levels, and in both treatments there was no reduction in aflatoxin B_2 levels, suggesting that the vinyl ether double bond is the primary site of reaction. Feeding trials with rats and ducklings on the ozonised peanut meal showed a lowering in available lysine and PER values.

1.3.3 Bisulphite Treatment.

Sodium bisulphite has been found to degrade aflatoxins B_1 and G_1.[49,50] The reaction is first order in bisulphite, and at 55 °C, in a buffer of pH 5.5, with 0.05 M K_2SO_3, the half-life of aflatoxin B_1 is 21 hours. Aflatoxin G_1 has been found to react somewhat faster than aflatoxin B_1 under these conditions.



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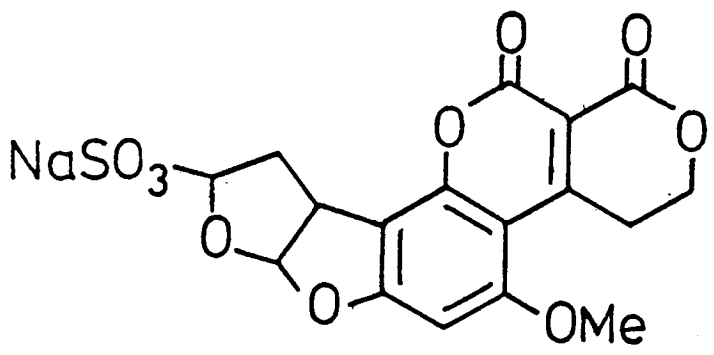


(26)

Transferring these observations into practice,[51] Moerck et al. were successful in reducing aflatoxin B_1 and B_2 levels in naturally contaminated corn (235 ppb) to below the FDA guidline of 20 ppb by treatment at a moisture level of 20% for 24 hours at ambient temperature with 2.0% aqueous sodium bisulphite.

More forcing conditions were required when highly contaminated corn was used. To obtain complete destruction of aflatoxin B_1,[52] the corn was soaked in a 10% bisulphite solution for 72 hours, then removed and sealed in plastic bags at 50 °C for 21 days. Aflatoxin B_2 was found to respond unpredictably to the treatment, and at best only 50% destruction was achieved.

The major reaction products of aflatoxins B_1 and G_1 with bisulphite, named aflatoxins B_1S (25) and G_1S (26) respectively, have been isolated,[53] and B_1S has been identified by fast atom bombardment mass spectroscopy (FAB MS), UV, IR, and proton nmr. FAB MS[54] gave a molecular weight of 416 for aflatoxin B_1S sodium salt, which is a light yellow, highly fluorescent, water-soluble compound. The UV spectrum was very similar to that of aflatoxin B_1, indicating that the coumarin moiety had remained intact. The IR spectrum lacked the band due to the vinyl ether in aflatoxin B_1, and an intense absorbance at 1210 cm^{-1} was attributed to a sulphonate group. Proton nmr also indicated that the bisdihydrofuran moiety had been altered. The above data therefore suggested that aflatoxin B_1S is the bisulphite addition product (25).

1.3.4 Formaldehyde Treatment.

The use of formaldehyde as a reagent for reducing the aflatoxin content in peanut meal was reported by Mann *et al.*[55] The meal, containing 110 ppb aflatoxins, and 30% moisture was treated with 2% formaldehyde at 100 °C for 120 minutes. The process was later found to be enhanced by the inclusion of 2% calcium hydroxide.[56]

More recently, Piva obtained a 98% reduction in aflatoxin B_1 in a sample of groundnut meal,[57] initially at 1000 ppb, by treatment at 17% moisture, 130 °C with 4% calcium hydroxide and 1% paraformaldehyde for 30 minutes. Being powders, the reactants have the advantage of being simpler to handle, but it was found that particle size and mixing efficiency became critical factors.

1.3.5 Alkaline Treatments.

1.3.5.1 Sodium hydroxide.

The removal of aflatoxins from peanut meal using sodium hydroxide has been reported.[47] The meal was heated to 100 °C with a 5.8% solution of sodium hydroxide for 90 minutes, and after drying was found to be non-toxic in feeding experiments with ducklings and rats. Mann *et al.* noticed[55] a reduction in aflatoxin content from 100 ppb to 4 ppb in peanut meal, using 3% sodium hydroxide at 100 °C for 120 minutes.

Another detoxification process used 6% sodium hydroxide during the pelleting of naturally aflatoxin-contaminated groundnut meal.[58] However, no better than 69% elimination of aflatoxins was obtained.

Moerck et al.[51] found that a 2% solution of sodium hydroxide was successful in reducing aflatoxin levels. The moisture content of yellow dent corn containing 200 ppb aflatoxin B_1 and 35 ppb aflatoxin B_2 was adjusted to 20%, and treatment with 2% sodium hydroxide solution for 24 hours reduced both B_1 and B_2 levels to below the detection limit.

Likewise, Lakshmirajam et al.[59] achieved reductions of 90% and 81% in aflatoxin levels of contaminated groundnut cake and maize respectively, after treatment with 2% sodium hydroxide and autoclaving for 1.5 hours. Feeding of the treated meals to broiler chicks did not significantly depress the growth response.

It may be presumed that base catalysed degradation of the coumarin system is involved in the above detoxification processes.

1.3.5.2 Calcium hydroxide.

Mann et al. have reported[55] the use of 2% calcium hydroxide for reducing the aflatoxin level in peanut meal from 99 to 12 ppb. Subsequent use of calcium hydroxide gave erratic results, and Esproy stated[60] that a mean particle size of 50 microns or less was required for successful reduction of aflatoxins levels in pelletised copra, peanut and cottonseed meals. Codifer et al.[56] found that heating a peanut meal sample containing 570 ppb total aflatoxins with 2% calcium hydroxide at 117 °C and 15% meal moisture, for one hour, reduced the toxin level to 26 ppb.

Finally, the manufacture of tortillas requires corn to

be treated with aqueous calcium hydroxide to loosen the husks. Unfortunately, only ca. 40% reduction in aflatoxin was seen when contaminated corn was treated in this manner.[61]

1.3.5.3 Methylamine.

The use of volatile bases may be preferable to solutions, because there will be better penetration of the reagent throughout the batch being treated.

Dollear et al.[47] added methylamine as a 40% aqueous solution to contaminated peanut meal and heated it in a sealed reactor at 100 °C for 90 minutes, reducing the aflatoxin content to less than 5 ppb. Mann et al. also found methylamine an effective treatment for reducing aflatoxins:[53] cottonseed meal containing 334 ppb aflatoxins was treated with 2% methylamine at 100 °C and 15% moisture for 30 minutes; 17 ppb of the toxins were found in the product, and this could be reduced to non-detectable levels by the inclusion of 1% sodium hydroxide. Feeding trials with rats indicated a 28% reduction in PER value for the methylamine-treated meal.

Park et al. have investigated[62] the methylamine-calcium hydroxide decontamination of aflatoxin-contaminated peanut meal, which has been used on pilot scale in Senegal. ^{14}C-Labelled aflatoxin B_1 was added to naturally contaminated peanut meal, and treated with 2% calcium hydroxide and 0.5% methylamine, using a moisture level of 25%, at 100 °C for one hour. The process resulted in a 94-100% reduction in aflatoxin levels. After air-drying and extraction by

solvents of increasing polarity, the majority of the radioactivity (69-80%) remained associated with the meal. Enzymatic digestion released 12% of the original radioactivity as aflatoxin B_1 and 27% as water solubles. In addition, a large number of by-products, fluorescent and non-fluorescent, were visible by tlc and hplc. Tests using animal and bacterial cells, and chick embryos, showed that some of these by-products were harmful, although inferior in toxicity to aflatoxin B_1.

However, more information on the chemical structure and toxicology of the by-products of these methylamine treatments is clearly desirable.

1.3.5.4 Ammonia treatment.

Ammonia has been used to destroy aflatoxins in various feedstuffs, either in its gaseous form, or as ammonium hydroxide solution, and at various temperatures, pressures, moisture levels and reaction times.

The aflatoxin content of contaminated peanut meal has been reduced from 111 ppb to 5 ppb by Dollear et al.,[47] who used anhydrous ammonia at 42 psi in an iron pressure vessel, held at 68 °C for 15 minutes. The PER value of the meal was somewhat reduced in the process.

Similar results have been achieved by Mann et al.[55] for cottonseed meal, using similar conditions. Further studies into ammoniation of peanut and cottonseed meals at elevated temperatures and pressures have generally found good reductions in aflatoxin levels.[59,63-68]

Peanut meal has been ammoniated on an industrial scale

in both Senegal and France.[69-71] The process used by a plant in Dakar in 1979 involved heating the meal to 80 °C, altering the moisture content to 5-15%, injecting gaseous ammonia at 2 bars, and allowing a reaction time of 20 minutes in the mixer. The process was later improved by the addition of 0.3-0.6% formaldehyde as an antibinding agent, and in 1984 the total throughput in Senegal was approaching 600,000 tonnes a year.

Recently, workers in India[72] have achieved up to 85% destruction of aflatoxins in peanut products using 20% urea and 2% soyabean flour (a urease source) as a cheaper source of ammonia.

Good results have been obtained feeding ammoniated aflatoxin-containing peanut meal to ducklings,[73] and hens and chicks.[74] McKinney et al. have studied the effect of feeding lactating cows aflatoxin-containing cottonseed and cottonseed meals that had been treated by ammoniation.[75] There was no aflatoxin M_1 detectable in the milk of cows fed ammoniated meals, indicating that the ammoniated feed is safe for ruminants.

Similarly, Price et al.[76] have found a 90% reduction in aflatoxin M_1 in the milk of cows fed aflatoxin-contaminated whole cottonseed that had been stored in a large polythene bag with ammonium hydroxide for 21 days, over those fed the untreated cottonseed.

Ammoniated cottonseed has also been used effectively as a protein supplement for laying hens.[77,78]

The approach taken by Brekke et al. to detoxify

aflatoxin-contaminated corn[79] involved the use of aqueous ammonia at atmospheric pressure. Increasing moisture and temperature was found to favour detoxification. For instance, to reduce contamination from 1000 ppb to 20 ppb using 1.5% ammonia and 17.5% corn moisture required 42 days at 10 °C, 8 days at 25 °C, or 2 days at 40 °C.

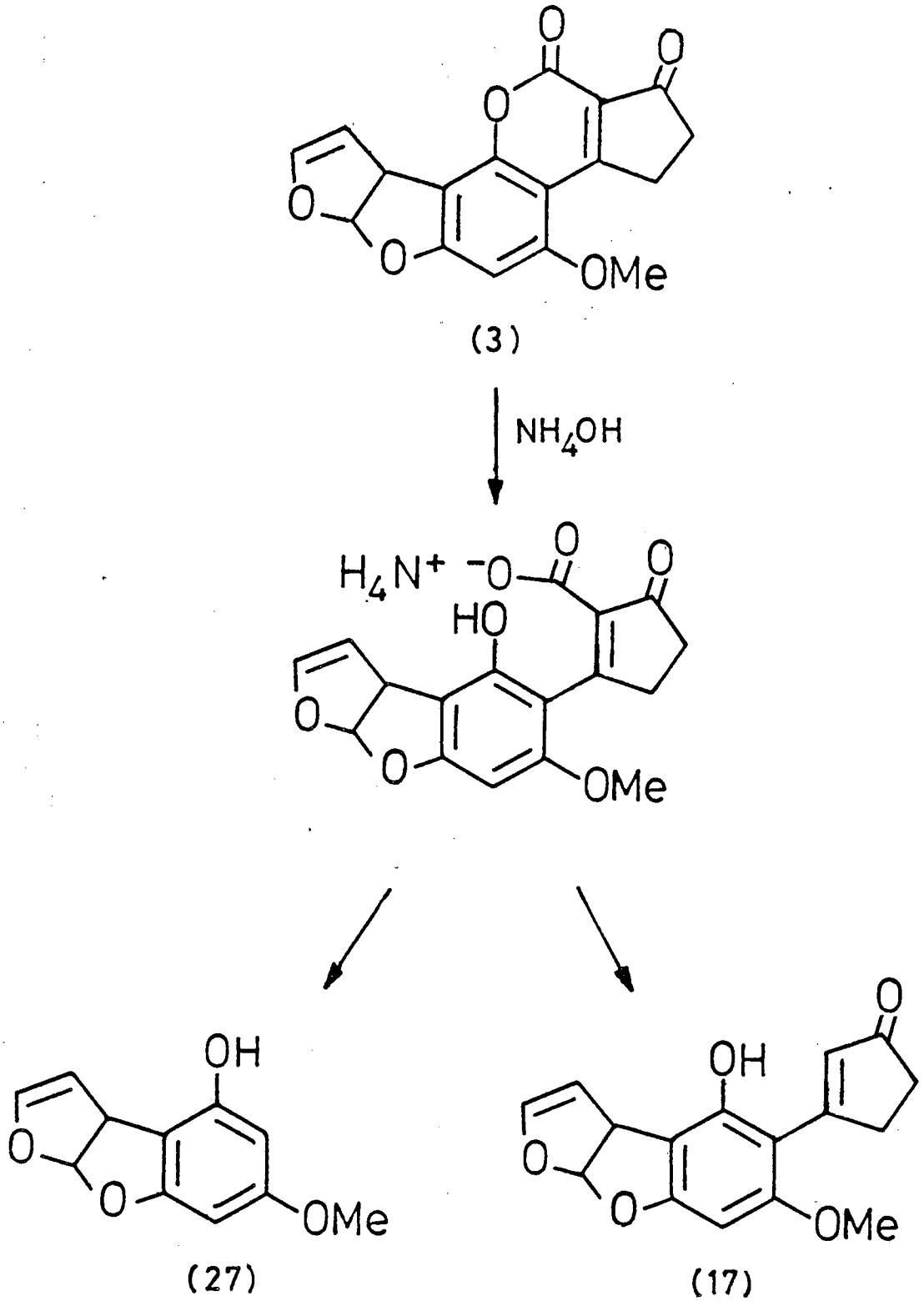
The use of ammonia gas has also been studied by the same workers,[80] and found to be equally as effective. An ammonia-air mixture was recycled through a bed of corn containing 17% moisture, at 25 °C, until there was an even ammonia distribution. The reactor was then sealed and held at 25 °C for 14 days, reducing the aflatoxin B_1 content from 1000 ppb to 10 ppb.

A farm-scale application of this process has been described by Bagley,[81] and other workers have produced similar results in the treatment of aflatoxin-containing corn with ammonia.[51,59]

The results of toxicity studies on corn ammoniated to reduce aflatoxins have been reviewed,[82] and the results are encouraging.

Brekke et al. showed[83] that rainbow trout fed a diet containing 25% of a sample of corn contaminated with 180 ppb aflatoxin suffered a 96-98% hepatoma incidence. The occurence of hepatomas was reduced to less than 3% in trout fed contaminated corn which had previously been ammoniated.

In addition, the metabolic fate of aflatoxin-related reaction products from corn following ammoniation has been investigated.[84] Using ^{14}C-labelled aflatoxin B_1 as a tra-



Scheme (3)

cer, it was found that the radioactivity was excreted more quickly in rats fed corn ammoniated to reduce aflatoxins than in those fed the untreated corn. Also, the concentration of radioactivity in the blood and liver was much less in those rats fed the ammoniated corn.

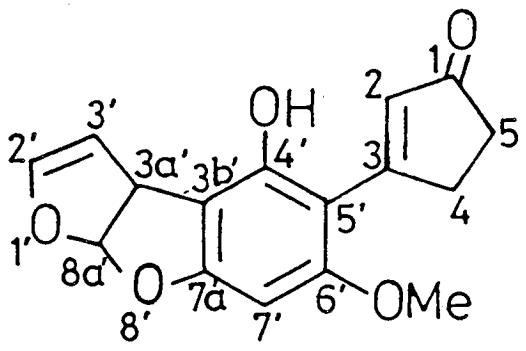
Norred and Morrisey have demonstrated the safety of ammoniated corn in long-term feeding trials with Fischer 344 rats.[85] Hughes et al. have also fed such corn to White Leghorn layer breeders with good results.[86,87]

In other studies using rats, Schroeder et al.[88] determined the covalent binding index (CBI) to rat liver DNA (a measure of carcinogenicity) of an ammoniated sample of aflatoxin-containing corn. Ammoniation was found to reduce the CBI by at least 20 times.

Since showing promise as a means of detoxifying aflatoxin-contaminated products, the ammoniation process has been investigated in order to determine the degradation products, and the nature of their binding to the substrate.

In a model reaction,[20] Lee et al. heated aflatoxin B_1 with concentrated ammonium hydroxide in a sealed Parr bomb at 100 °C for one hour. The major component, isolated by chromatography, was a non-fluorescent phenol of molecular weight 286, lacking the lactone carbonyl of aflatoxin B_1. The structure, based on further spectrometric and chemical analyses, was proposed, and the compound named aflatoxin D_1 (17), as it is formally derived by decarboxylation of the lactone ring-opened form of aflatoxin B_1.

In the same year, Kiermeir and Ruffer reported[89]



Chemical Shift (δ)	Carbon
31.9	4
34.2	5
47.6	3a'
55.8	OMe
86.6	7'
103.1	3'
106.1	3b' or 5'
106.7	3b' or 5'
111.5	8a'
131.5	2
144.1	2'
151.3	4'
158.6	7a'
159.5	6'
170.9	3
208.6	1

Table (1): ^{13}C nmr spectrum of aflatoxin D_1, dissolved in d_6-DMSO.

isolating the same compound from the degradation product mixture of aflatoxin B_1 in a diethanolamine buffer, and also from treatment with sodium hydroxide.

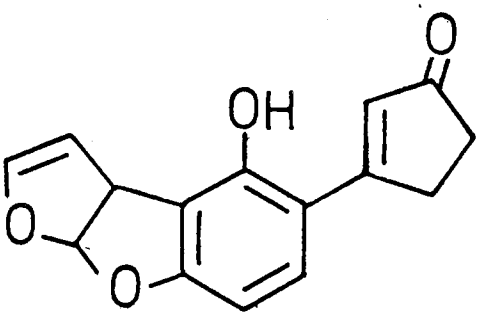
The ^{13}C nmr spectrum [table (1)] of aflatoxin D_1 has been reported,[90] and is in accord with the proposed structure.

Aflatoxin D_1 has also been prepared[91] in 28% yield by heating aflatoxin B_1 with concentrated ammonium hydroxide in a sealed flask at 50 °C for 14 days.

In separate mutagenicity tests, Lee et al.[92] and Schroeder et al.[88] found aflatoxin D_1 to be of the order of 450 times less mutagenic than aflatoxin B_1. Chick embryo trials carried out by Lee et al., also indicated a decrease in toxicity of 18-fold.

Continuing Lee's work, Cucullu et al.[93] isolated another product from the reaction of aflatoxin B_1 and concentrated ammonium hydroxide. This compound was another, less polar, non-fluorescent phenol, of molecular weight 206, lacking the cyclopentenone ring of aflatoxin B_1. This compound (27), previously prepared by Buchi[94] as a key intermediate in an aflatoxin B_1 synthesis, was also postulated as arising from a ring-opened form of aflatoxin B_1. These transformations are summarised in scheme (3).

Two further minor products were obtained[95] by microsublimation of an unpurified sample of aflatoxin D_1, and subjected to mass spectral analysis. One of the products had a molecular ion of m/e 236, and the other had m/e 256. The latter is consistent with a demethoxylated aflatoxin D_1



(28)

(28). In fact, such a compound was also reported[89] by Kiermeir and Ruffer in their reaction mixture described earlier.

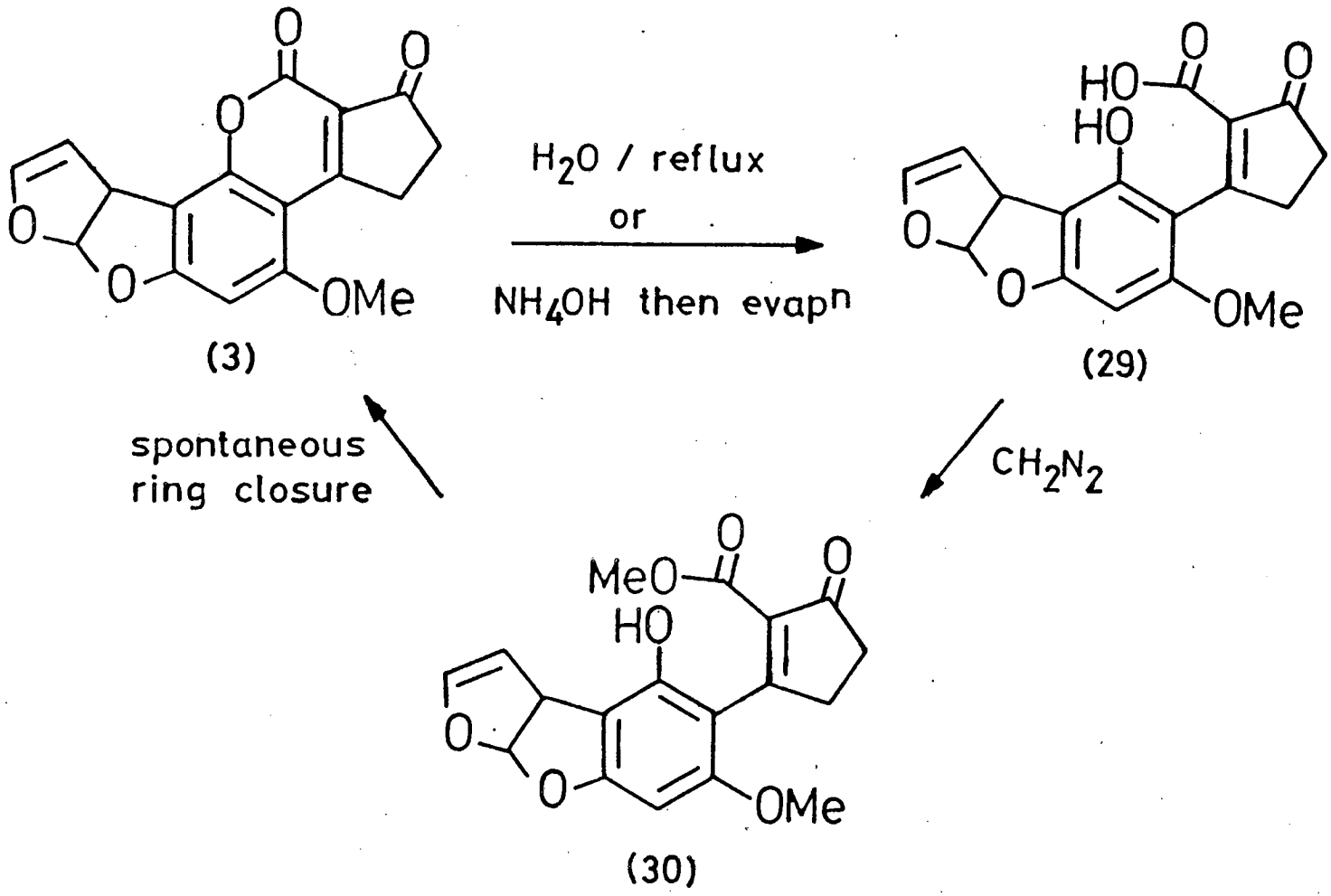
Recently, pure aflatoxin B_1 was ammoniated[96] using gaseous ammonia at 40 psi and 100 °C for 30 minutes. In this case, 20% of the degraded toxin was accounted for as the molecular weight 206 compound (27), but no aflatoxin D_1 was detected, and only 1% of the original aflatoxin B_1 remained.

The first step in the degradation is likely to be the base-induced lactone ring opening of aflatoxin B_1. It has been noticed[37] that aflatoxin fluorescence, attributable to the coumarin moiety, disappears in alkaline solutions, but reappears upon acidification.

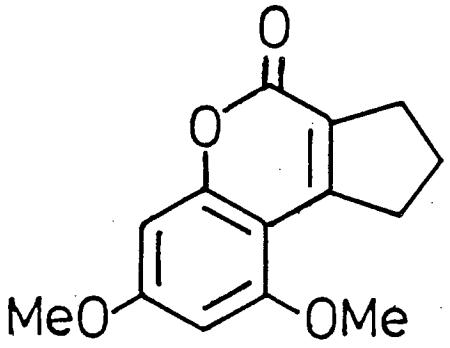
Beckwith et al. have reported[97] that the UV maximum of aflatoxin B_1 in concentrated ammonium hydroxide solution was at 363 nm, the same as for B_1 in methanol, but with a much reduced extinction coefficient. Evaporation of the alkaline solution, and redissolving in water, produced a solution which had a UV maximum as a broad band at 325 nm. Subsequent acidification resulted in the regeneration of aflatoxin B_1.

This corresponds with a report by Coomes,[98] which states that the substituted o-coumaric acid (29) produced by refluxing aflatoxin B_1 in water, had a UV maximum at 324 nm, undergoing a bathochromic shift of 36 nm to 360 nm, on addition of alkali.

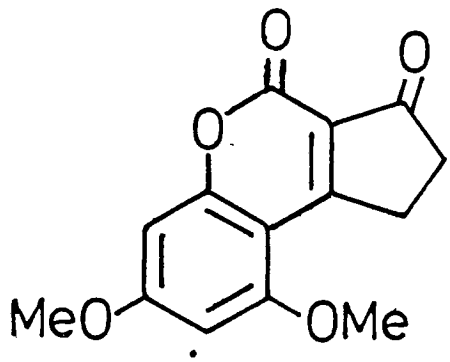
Vesonder et al.[99] reacted aflatoxin B_1 with concentra-


H2O / reflux
or
NH4OH then evapn
spontaneous
ring closure
CH2N2
(3)
(29)
(30)

Scheme (4)



(31)



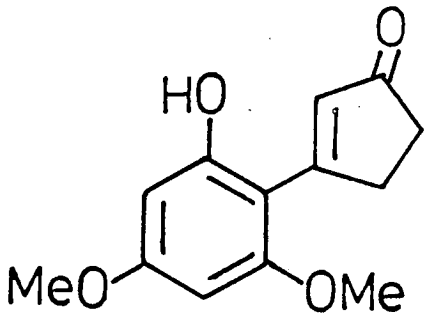
(32)

ted ammonium hydroxide at room temperature for 18 days. After taking the mixture to dryness, it was extracted with acetone. The product mixture had a mass spectrum with prominent ions at m/e 330 and 312, and IR absorbances at 3320, 1750 and 1620 cm^{-1}, consistent with the free beta-keto acid (29). Reaction with diazomethane gave a product with a new IR carbonyl band at 1700 cm^{-1}, which was observed to rapidly ring-close to generate aflatoxin B_1 [scheme (4)]. This must have been due to the formation of the methyl ester (30) of the substituted o-coumaric acid, formation of which provides a better leaving group for ring-closure.

Beckwith et al.[100] have reported that radiolabelled aflatoxin B_1 binds covalently to corn grain flour components when treated with ammonia. They found that the covalent binding did not change the spectral properties of the aflatoxin B_1 chromophore, and hence proposed that aflatoxin B_1 binds to the corn via the bisdihydrofuran system. The marked lack of destruction of aflatoxin (40-50%) when reacted with corn starch and ammonium hydroxide, was taken as indirectly implicting the protein as providing the active sites for interaction with the corn grain flour.

However, model compounds lacking the bisdihydrofuran system have been found to be effectively degraded by ammoniation.

Using model coumarins (31) and (32), available in relatively large quantities by synthesis,[7] Grove and co-workers have studied the ammoniation process.[101] Compound



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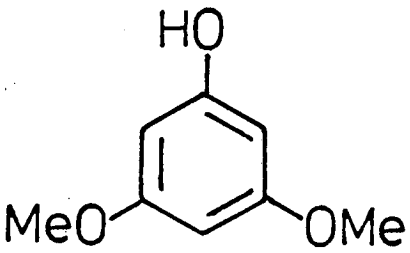
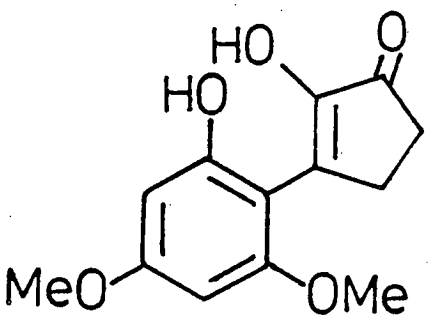


(34)



(35)

(31), lacking the ketone carbonyl, was found to be inactive to ammonia, whether in contact with corn germ or not.

Coumarin (32) is a better model for aflatoxin B_1, since it lacks only the bisdihydrofuran moiety, which is stable to ammonia (at least in the absence of meal). Reaction of this compound with concentrated ammonium hydroxide at 50 °C for 14 days resulted in 53% yield of (33), analagous to aflatoxin D_1, and 6% of 3,5-dimethoxyphenol (34), analagous to the molecular weight 206 compound. 28% Of the starting material was recovered.

When the reaction was repeated at lower temperature (37 °C for 20 days), low yields (2-10%) of (33), (34), and the novel compound (35) were obtained. The aflatoxin D_1 analogue (33) was found to be stable to ammonium hydroxide, but (35) was in part degraded to (34). Compound (34) was stable to ammonium hydroxide at 37 °C for 13 days, but was completely degraded in the presence of corn germ. Likewise, the coumarin (32) decomposed to give no isolable products upon ammoniation in the presence of corn germ.

Aflatoxin D_1 has been detected in ammoniated corn by tandem mass spectrometry,[91] and in ammoniated peanut and cottonseed meals by the fluoresence of its acetate.[67] Only small amounts (0.36% and 0.37%) of aflatoxin D_1 and aflatoxin B_1 were observed.

The destruction of aflatoxins in ammoniated products has also been followed by radioactivity, after spiking the samples with ^{14}C-labelled aflatoxin B_1.[65,66,88,100]

Cottonseed meal naturally contaminated with 4000 ppb

aflatoxins was spiked by Park et al.,[66] with ^{14}C-labelled aflatoxin B_1, and treated with 4% ammonia, 40 psi at 100 °C for 30 minutes, reducing the toxin content to 4 ppb. The meal contained 86% of the initial radioactivity after drying, and 25% was extracted with methylene chloride. 5% Was extractable into methanol, and enzymatic digestion, followed by methylene chloride extraction of the residue, liberated a further 19% of the radioactivity, leaving 37% irreversibly bound to the meal matrix.

It is noteworthy that the radioactivity to weight ratio was by far the highest for the methylene chloride extract of the enzymatic digest residue, and that in a subsequent study[102] of the mutagenic potential (Ames test) of the various fractions, only this extract gave a positive response. However, 180 micrograms of the extract was required to give the same response as for 0.005 micrograms of aflatoxin B_1.

Finally, while the amount of aflatoxin D_1 that has been detected in ammoniated meals is low, and although the importance of aflatoxin D_1 and the molecular weight 206 compound in the ammoniation process has been questioned, it is likely that a general oxidative process, as described by Grove[101] for model compounds, is occuring to some extent, in addition to the covalent binding to protein fractions favoured by Beckwith.[100]

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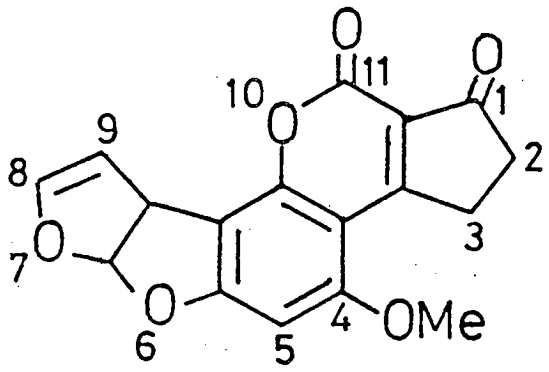
CHAPTER 2

AMMONIATION OF AFLATOXIN B_1 : INITIAL INVESTIGATIONS AND SYNTHETIC STUDIES.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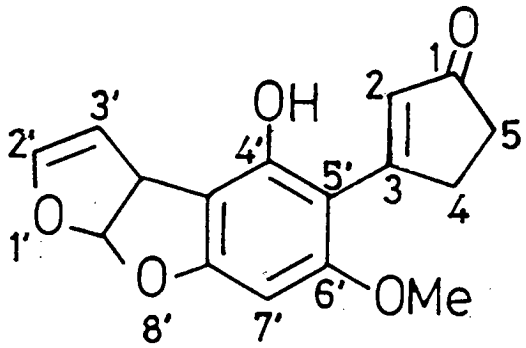
(1)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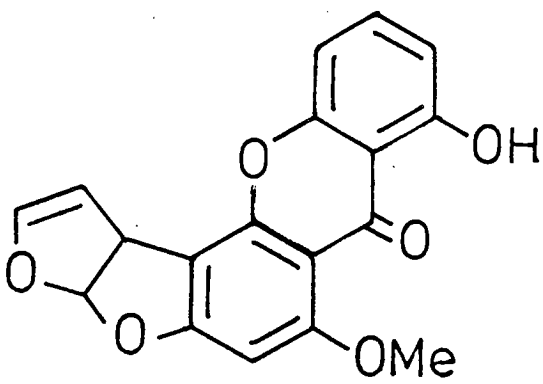
(2)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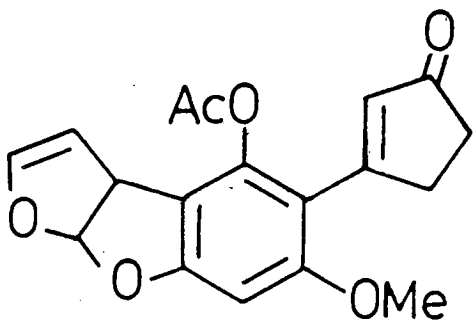
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(4)

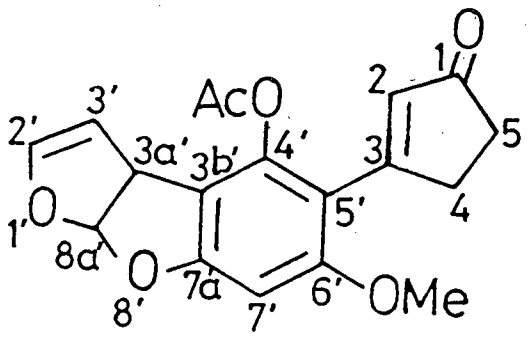
2.1 INTRODUCTION.

The initial aim of this research was to isolate and identify the products derived from the treatment of aflatoxins with ammonia, and to study the mechanism of their formation. Ultimately, the nature of the binding of the ammoniation products to meal matrices needs to be examined, but clearly, a good understanding of the degradation of the aflatoxins themselves by ammonia requires first to be obtained.

2.2 TREATMENT OF AFLATOXIN B_1 (1) WITH AMMONIUM HYDROXIDE. PRELIMIMARY STUDIES.

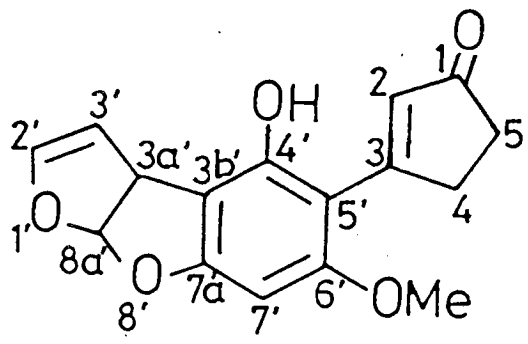
Initially, aflatoxin B_1 (1) was refluxed overnight in concentrated ammonium hydroxide solution. Analytical tlc of the product mixture showed the presence of aflatoxin B_1 and a more polar compound, corresponding to the reported[1] aflatoxin D_1 (2). When sterigmatocystin (3) was subjected to the same conditions, it was recovered unchanged, indicating that the bisdihydrofuran moiety, present in both sterigmatocystin and aflatoxin B_1, is stable to ammonium hydroxide.

In order to investigate prolonged heating with ammonium hydroxide, it was necessary to use a closed system. Aflatoxin D_1, produced from the reaction of aflatoxin B_1 with concentrated ammonium hydroxide in a sealed flask, kept at 56 °C for 14 days, was found to be insufficiently soluble in deuterated chloroform, acetone, or dimethyl sulphoxide for nmr analysis. Therefore, the sample was treated with pyridine and acetic anhydride, to form the



Chem. Shift (δ)	Multiplicity	No. H	Assignment
2.22	s	3	$O_2CC\underline{H}_3$
2.46	m	2	<u>H</u>-5
2.97	m	2	<u>H</u>-4
3.79	s	3	$OC\underline{H}_3$
4.47	dt, J 7.1, 2.2 Hz	1	<u>H</u>-3a'
5.14	t, J 2.5 Hz	1	<u>H</u>-3'
6.15	t, J 1.8 Hz	1	<u>H</u>-2
6.45	dd, J 2.7, 2.0 Hz	1	<u>H</u>-2'
6.46	s	1	<u>H</u>-7'
6.69	d, J 7.1 Hz	1	<u>H</u>-8a'

Table (1): 200 MHz proton nmr spectrum of aflatoxin D_1 acetate (4) in $CDCl_3$.



Chem. Shift (δ)	Multiplicity	No. H	Assignment
2.52	m	2	H-5
3.22	m	2	H-4
3.81	s	3	OCH_3
4.67	dt, J 7.1, 2.3 Hz	1	H-3a'
5.43	t, J 2.4 Hz	1	H-3'
6.20	s	1	H-7'
6.48	t, J 1.7 Hz	1	H-2
6.50	dd, J 2.7, 2.1 Hz	1	H-2'
6.73	d, J 7.2 Hz	1	H-8a'

Table (2): 200 MHz proton nmr spectrum of aflatoxin D_1 (2) in $CDCl_3$ - CD_3OD.

acetate[1] (4), which was soluble in chloroform. The acetate was also isolated in 36% yield by acetylation of the crude mixture following treatment of aflatoxin B_1 with concentrated ammonium hydroxide, and subsequent preparative tlc. The proton nmr [table (1)] of this compound was consistent with the proposed structure (4), as described below.

The presence of the dihydrofurobenzofuran moiety was evident from the coupling pattern.[2] The doublet of triplets at 4.47 ppm, due to the benzylic proton, was the result of a coupling of 7 Hz to the acetal proton at 6.69 ppm, and couplings of 2 Hz, to H-3' at 5.14 ppm, and allylic coupling to H-2', which was present as a doublet of doublets at 6.45 ppm. The presence of an olefinic proton as a triplet at 6.15 ppm which collapsed to a singlet on irradiation of the methylene multiplet at 2.97 ppm, and the concomitant simplification of the multiplet at 2.46 ppm into an AB coupling pattern, confirmed the presence of the cyclopentenone system; the methylenes at 2.46 and 2.97 ppm being α- and β- respectively to the carbonyl function.

Further experimentation revealed that aflatoxin D_1 would dissolve in a mixture of deuterochloroform and deuteromethanol, giving a proton nmr spectrum [table (2)] similar to that of the acetate (4).

The molecular weight 206 compound[3] (MW206) (5) was also found to be present by analytical tlc of aflatoxin B_1 ammoniation mixtures, showing up as a characteristic orange spot following spraying with fast blue B.[3] However, since it was present only in small quantities, larger scale

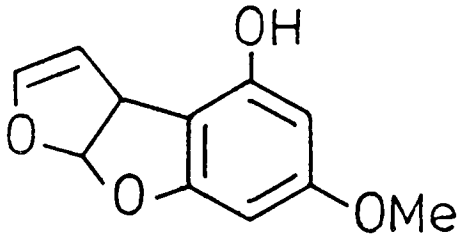
ammoniation of aflatoxin B_1 was carried out in a sealed flask at 50 °C for 21 days. In addition to aflatoxin D_1 (57%) and aflatoxin B_1 (15%), MW206 was isolated from the product mixture by preparative tlc, in 9% yield. There was now sufficient material for proton nmr spectroscopy, and the resulting spectrum was indistinguishable from that of the racemic compound, available by synthesis[4] (see section 2.4).

During these separations it was evident that there were no other significant products present in readily isolable quantities.

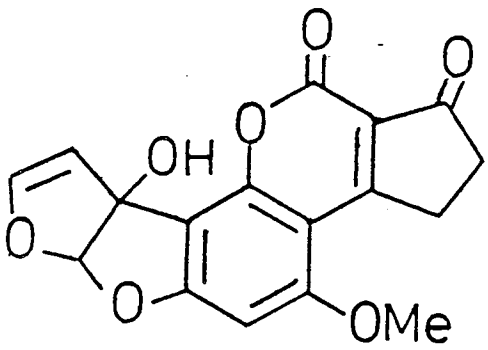
Visualisation of the weakly ultra-violet absorbing products on tlc was hampered by the proximity of bright blue fluorescent residual aflatoxin B_1, and this fact, combined with the limited amount of aflatoxin B_1 available for experimentation, made the isolation of pure degradation products difficult.

It was clear therefore, that the detection and identification of MW206 and aflatoxin D_1 would be greatly assisted by the use of reliable standards. Synthesis of these compounds would be desirable since, in addition to providing pure reference compounds, it would also act as unequivocal proof of structure. There is also a need for rigorous biological testing of these compounds, and synthesis would provide a means of obtaining quantities of material sufficient for these tests.

The remaining part of this chapter therefore describes studies arising from approaches to the synthesis of these



(5)



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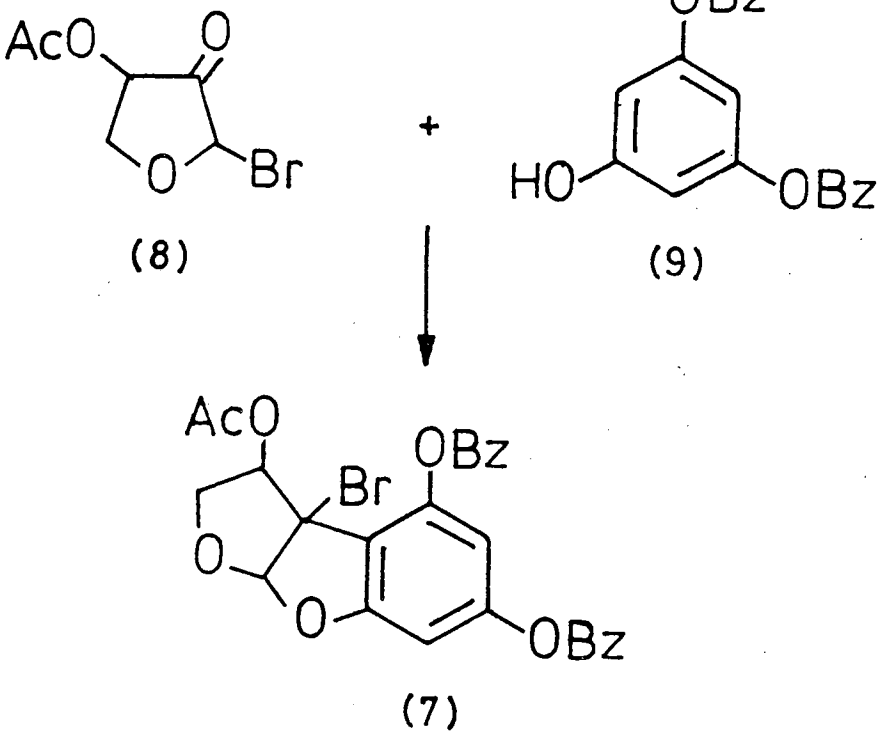
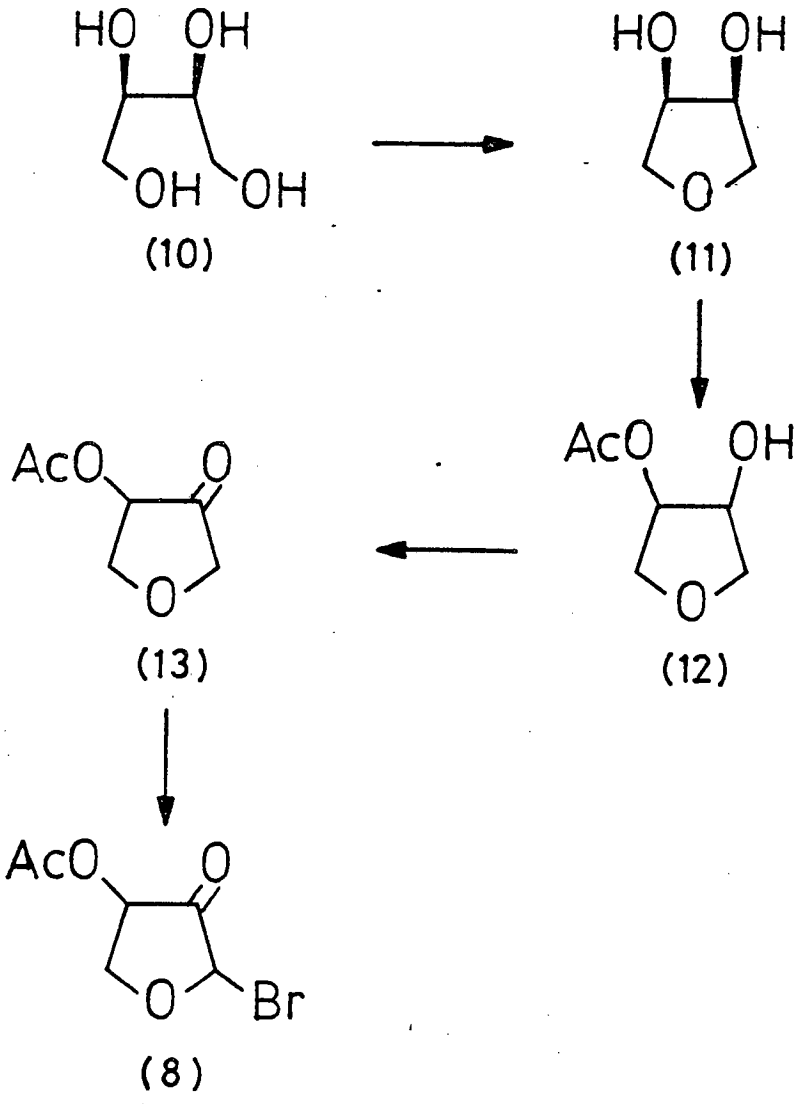



HO OH
OH OH
(10)
HO OH
O
(11)
AcO OH
O
(12)
AcO O
O
(13)
AcO O
O Br
(8)

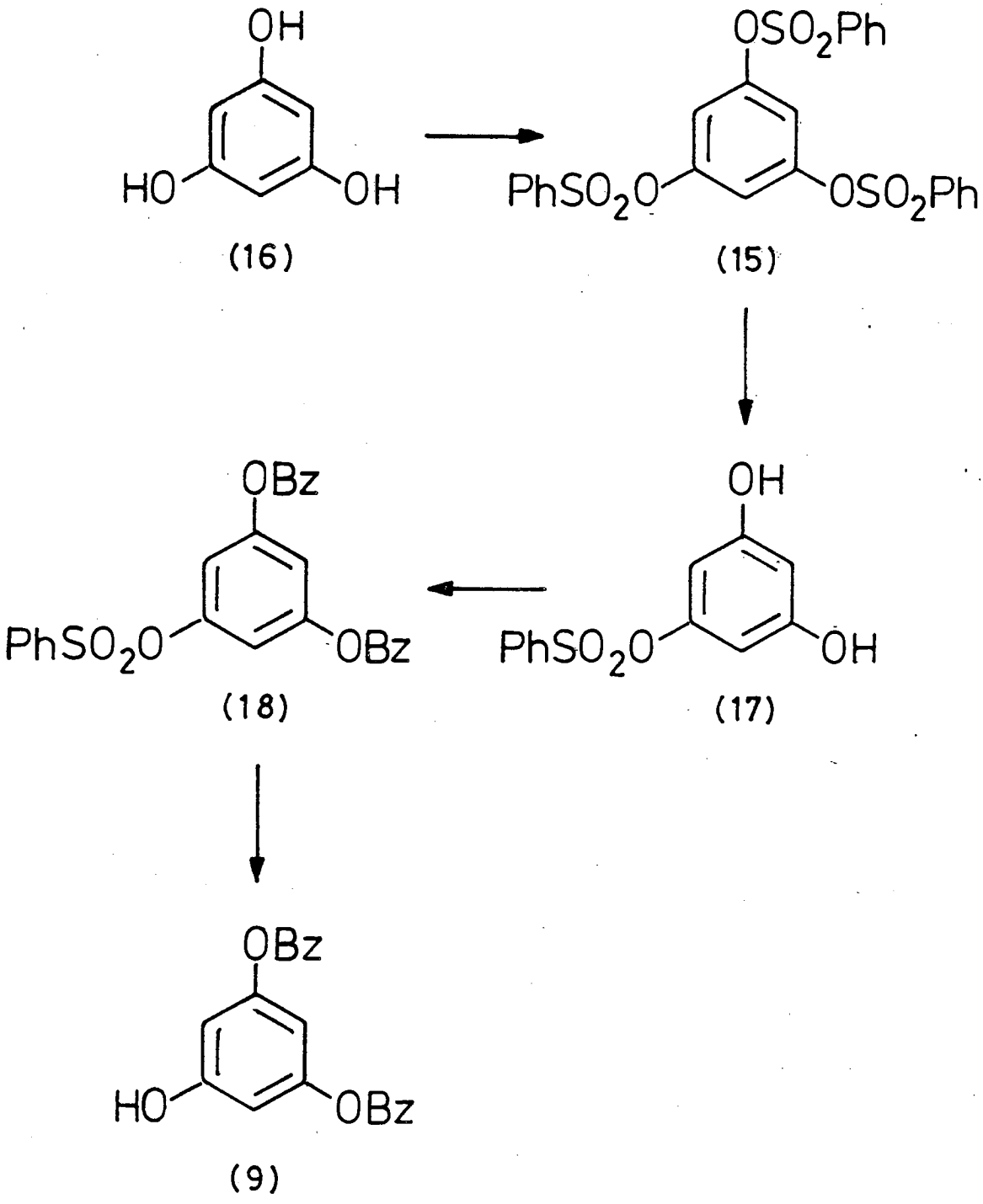
compounds.

2.3 ATTEMPTED SYNTHESIS OF MW206 (5).

MW206 (5) has previously been prepared by Buchi[4] as a key intermediate in the synthesis of aflatoxin B_1. However, more recent work[5] by the same author on the synthesis of aflatoxin M_1 (6) appeared to provide the opportunity for a novel synthesis of MW206. The key intermediate in this synthesis is the tricyclic bromide (7). The acetate group in this compound provides a suitable leaving group for formation of the required terminal double bond. In addition, the bromide can easily be converted into the tertiary hydroxyl function of aflatoxin M_1, and selective debenzylation followed by methylation provides a route to the correct regiochemistry about the aromatic ring. Thus, by replacement of the bromine by hydrogen, this route would provide a synthesis of MW206.

The bromide (7) was accordingly prepared, by the method of Buchi *et al.*,[5] using the hydrogen bromide catalysed condensation of the substituted tetrahydrofuranone (8) and phloroglucinol dibenzyl ether (9).

The furanone (8) was prepared as follows. *Meso*-erythritol (10) was cyclised by heating in the presence of acidic ion-exchange resin, the product distilling from the reaction mixture. The diol (11) thus formed was monoacetylated by the action of triethyl orthoacetate and a catalytic amount of trifluoroacetic acid, in refluxing tetrahydrofuran, followed by hydrolysis. This monoacetate (12) was then oxidised to (13) using pyridinium chlorochro-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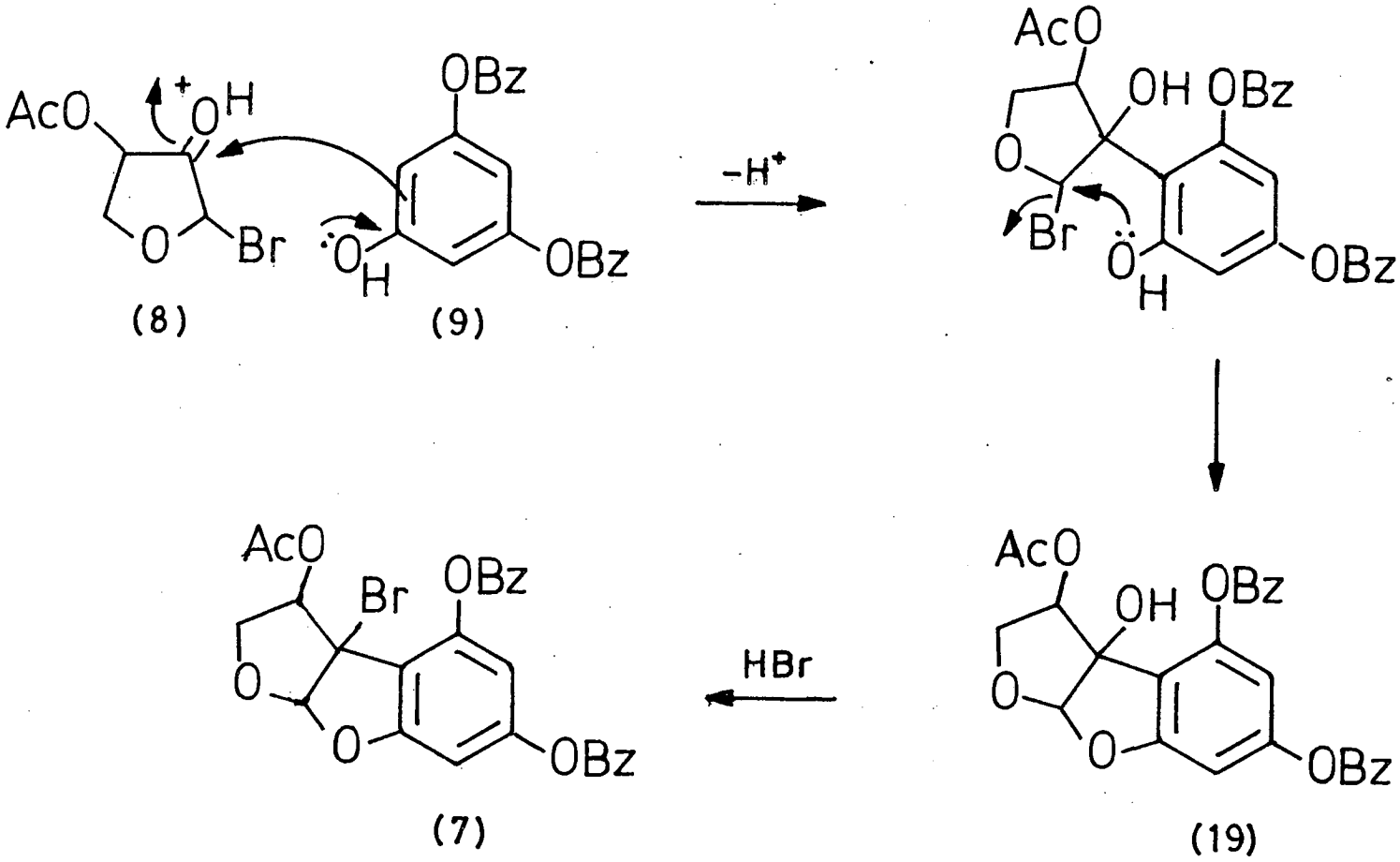
HO
OH
(16)
OSO2Ph
PhSO2O
OSO2Ph
(15)
OH
PhSO2O
OH
(17)
OBz
PhSO2O
OBz
(18)
OBz
HO
OBz
(9)

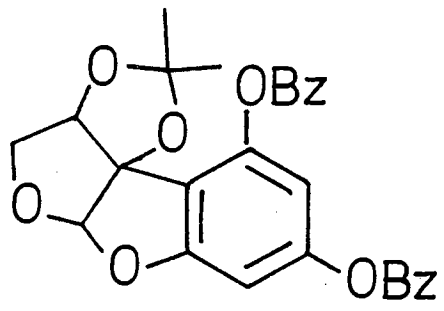
mate in methylene chloride, the resulting keto group providing the activation for N-bromosuccinimide bromination in the 2-position, to give bromide (8) which was unstable, even at -18°C, and had to be used within 24 hours.

Phloroglucinol tribenzenesulphonate (15) was prepared by the action of benzenesulphonyl chloride on phloroglucinol (16), with the addition of calcium hydroxide as base. The tribenzenesulphonate (15) was selectively hydrolysed in methanolic potassium hydroxide solution, to give the monobenzenesulphonate (17). This dihydroxy compound was dibenzylated using benzyl chloride and potassium carbonate in DMF; the product (18) being hydrolysed simply to produce phloroglucinol dibenzyl ether (9). Although this preparation of phloroglucinol dibenzyl ether (9) from phloroglucinol (16) was *via* a fairly lengthy route, the overall yield was good, and the separations trivial, which made it acceptable for larger scale preparations.

The substituted bromotetrahydrofuranone (8) and phloroglucinol dibenzyl ether (9) were then condensed by brief exposure to hydrogen bromide saturated methylene chloride. The yield of bromide (7) was rather disappointing (24%), and the product mixture often contained two other compounds which were isolated by column chromatography. The first of these compounds to be examined was more polar than the bromide (7) and had an IR absorbance at 3520 cm^{-1}, suggesting an alcohol. Mass spectrometry revealed the parent ion at *m/e* 448, and the compound analysed for $C_{26}H_{24}O_7$. The proton nmr spectrum was consistent with the



Scheme (1)



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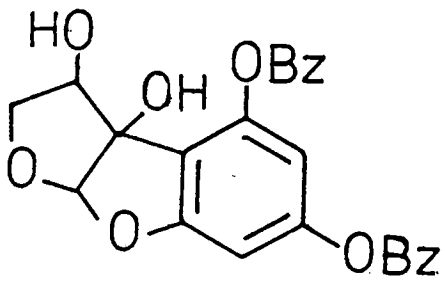


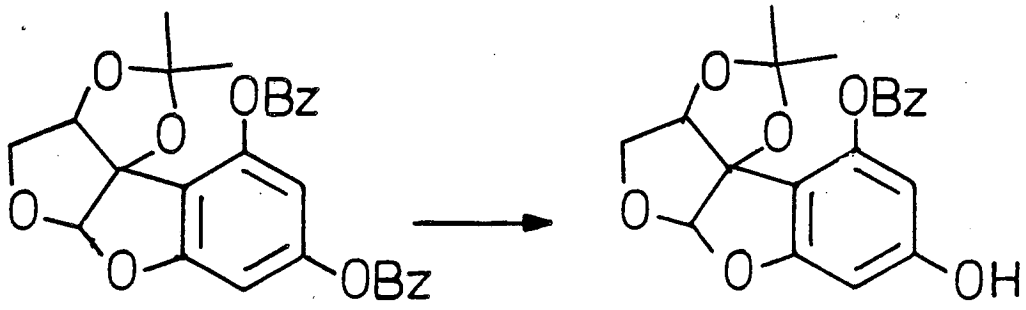
(21)

retention of the acetate moiety and both benzyl protecting groups, and an ABX system similar to that observed for the 2-methylene and 3-methine protons in the bromide (7) was also present. The evidence therefore suggests that this compound is the hydroxy-analogue (19) of the tricyclic bromide (7). The presence of this alcohol is in accord with the possible mechanism for the reaction, shown in scheme (1), where it appears as the initial condensation product, which may be transformed, by the subsequent action of hydrogen bromide, into the tricyclic bromide (7).

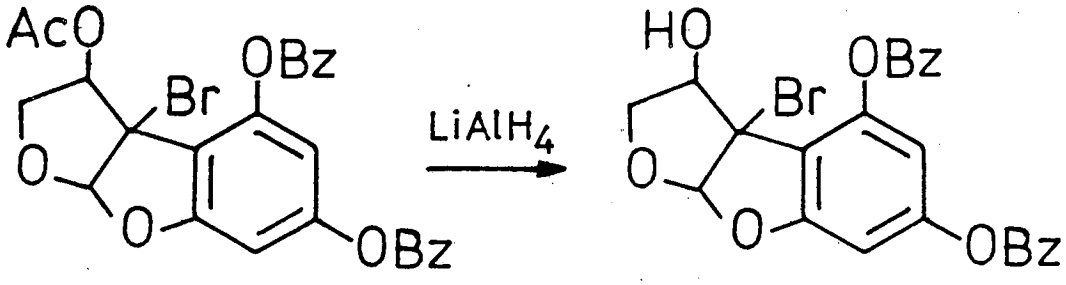
The other compound, which had a similar R_f to the bromide (7) on tlc in various solvent systems, appeared only in the acetone washings of silica columns which had been used to purify the product mixture. This compound was identified as the acetonide (20) by comparison of the proton nmr and mass spectra with those of the known compound,[5] indicating the presence of the highly polar diol (21) among the products.

Attempts were made to improve the yield of this reaction by employing different acidic catalysts, since a contributory factor to the low yield may have been cleavage of the aromatic ether links by hydrogen bromide.

When hydrogen chloride was used in place of hydrogen bromide, condensation did occur to give a product with the same R_f and proton nmr as the tricyclic bromide. However, on mass spectral analysis, this product proved to be a mixture of the bromide and the corresponding chloride. Hydrogen bromide, generated during the condensation to form


O
OBz
O
O
O
OBz
O
OBz
O
O
O
OH

(20) (22)


AcO
OBz
Br
O
O
OBz
LiAlH4
HO
OBz
Br
O
O
OBz

(7) (23)

the alcohol (19), may therefore have been acting in competition with hydrogen chloride in the ensuing substitution step. Using trifluoroacetic acid as the condensing agent, and increasing the reaction time to 22 hours, the bromide (7) was isolated in 16% yield.

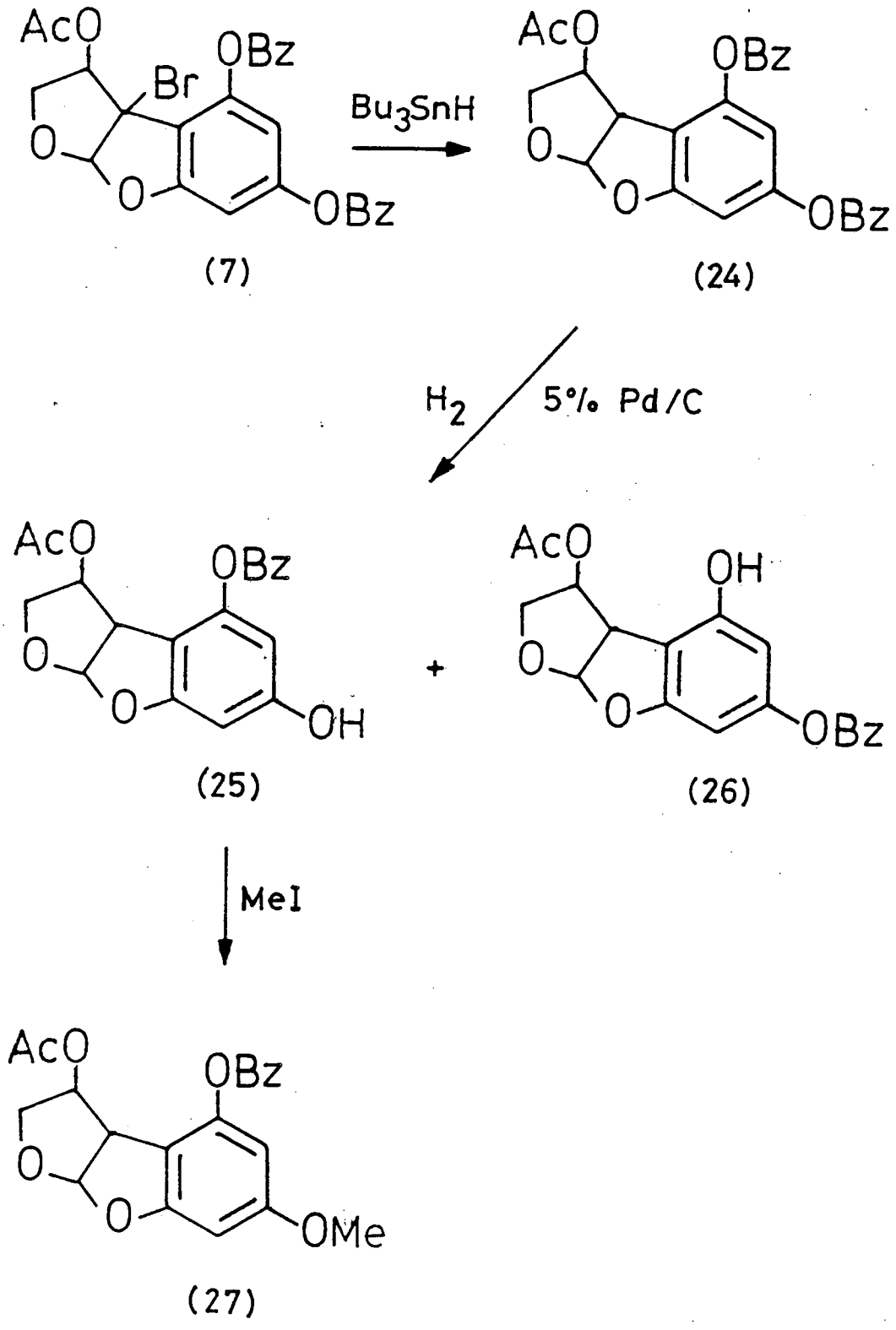
Having produced the tricyclic bromide in sufficient quantities, conditions for the reductive removal of the bromine were then investigated.

Catalytic hydrogenation is known to be of use in the reduction of benzyl bromides,[6] and this approach was tried first. There was also the possibility of performing a selective debenzylation at the same time, since Buchi has demonstrated[5] that controlled catalytic hydrogenolysis of the acetonide (20) can yield 88% of the monobenzyl compound (22) with the desired stereochemistry.

Unfortunately, catalytic hydrogenolysis, under a wide range of conditions, produced a mixture of products, among which there was no evidence for the loss of bromine.

The use of lithium alumimium hydride is documented for the reduction of alkyl halides.[6] However, refluxing the tricyclic bromide in ether with $LiAlH_4$ for one hour resulted only in the removal of the acetate group to give alcohol (23), as evidenced in the proton nmr by the lack of an acetate methyl signal, and the shift of the H-3 doublet from 5.89 ppm in the starting material to 4.62 ppm in the product. Prolonged reaction with $LiAlH_4$ yielded multi-component mixtures.

Replacement of the bromine atom by hydrogen was


AcO
OBz
Br
O
O
OBz
(7)
Bu_3SnH
AcO
OBz
O
O
OBz
(24)
H_2
5% Pd/C
AcO
OBz
O
O
OH
(25)
+
AcO
OH
O
O
OBz
(26)
MeI
AcO
OBz
O
O
OMe
(27)

finally achieved in 50% yield using tributyltin hydride[7] in refluxing benzene. The effect of this reduction was evident in the proton nmr spectrum of the product (24); the acetal proton, a singlet at 6.28 ppm in the case of the bromide, showing a characteristic doublet, with coupling 6 Hz, at 6.41 ppm.

It was thought appropriate at this stage to attempt a selective debenzylation in order to generate the desired regiochemistry at the benzene ring. Models indicated that the acetate function was sufficiently close to the 4-benzyloxy group to interact sterically and hopefully disfavour hydrogenolysis at that site. This indeed proved to be the case, although not to such a degree as in the example of the tricyclic acetonide (20) described earlier.

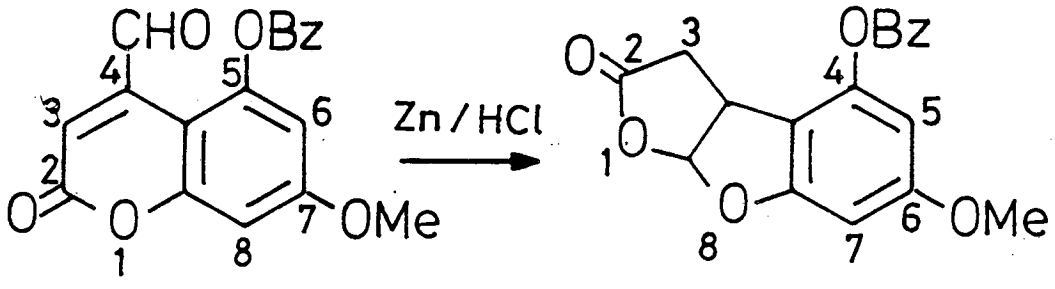
Controlled hydrogenolysis of the dibenzyl compound (24) using 5% palladium on carbon, in ethyl acetate, gave a 44% yield of a single regio-isomer, the residue consisting mainly of starting material and a minor amount of the other regio-isomer. The benzyl methylene in the major product was present in the nmr as a singlet at 5.07 ppm, whereas the methylene in the minor product was at 4.98 ppm. High field nmr analysis of the starting material shows two methylenes: a singlet at 4.97 ppm, and an AB quartet at 5.07 ppm. The coupling pattern in the latter arises from non-equivalence of the methylene protons, probably due to the proximity of the chiral bistetrahydrofuran moiety, suggesting that this is from the 4-benzyloxy group. The retention of the 5.07 ppm signal in the major product therefore suggests that

this is the desired 4-benzyloxy compound (25). Other evidence for this proposal comes from the fact that the 4-hydroxy compound should experience intramolecular hydrogen bonding between the phenol and acetate groups. This effect should reduce the electron donating capacity of the phenol and cause the aromatic protons to resonate at a higher frequency. The aromatic protons in the minor product gave doublets at 6.05 and 6.15 ppm, and those of the major product gave an AB multiplet at 5.99 ppm, consistent with the major isomer being the 4-benzyloxy compound (25). Finally, the minor isomer (26) was much less polar on tlc, which may have been due to hydrogen bonding.

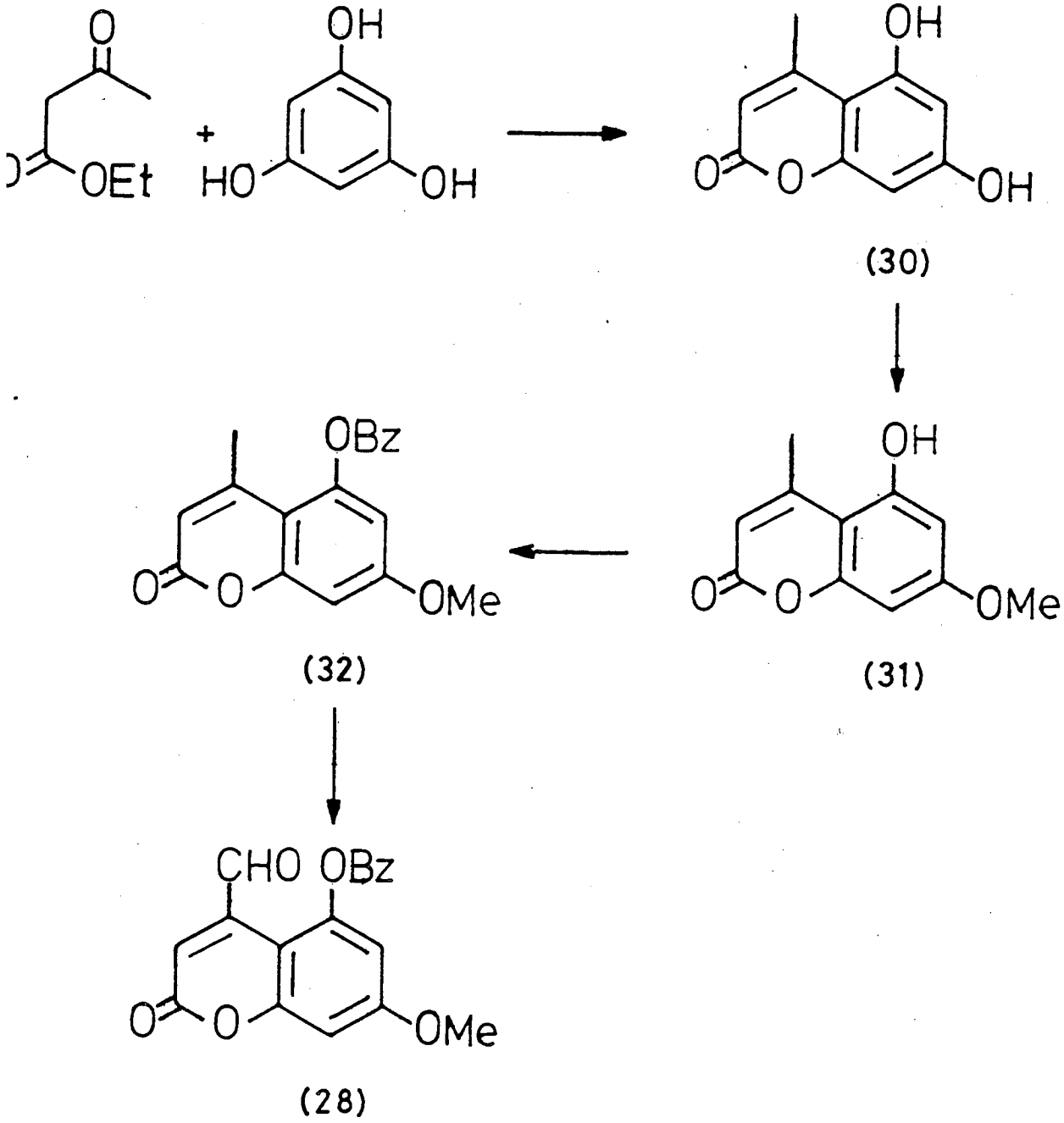
Having obtained the required phenol (25), this was methylated in quantitative yield by stirring for 4 days at room temperature with excess methyl iodide and potassium carbonate in acetone, to give the ether (27).

In an attempt to eliminate acetic acid, thus forming the terminal double bond, the methylated compound (27) was pyrolysed by heating in refluxing toluene. This produced no reaction, so the compound was subjected to flash vacuum pyrolysis.[8] The acetate was sublimed <u>in vacuo</u> (0.001 mm Hg) through a heated silica tube, and the products collected in a cold trap. At 500°C, the acetate sublimed through the apparatus unchanged. On increasing the furnace temperature to 600°C however, the compound suffered extensive break-down, and there was no sign of the desired acetate-elimination product in the mixture in the cold-trap.

At this stage it became apparent that this alternative



(28) (29)


O
OEt
+
HO
OH
OH
O
O
OH
(30)
OH
O
O
OMe
(31)
OBz
O
O
OMe
(32)
CHO
OBz
O
O
OMe
(28)

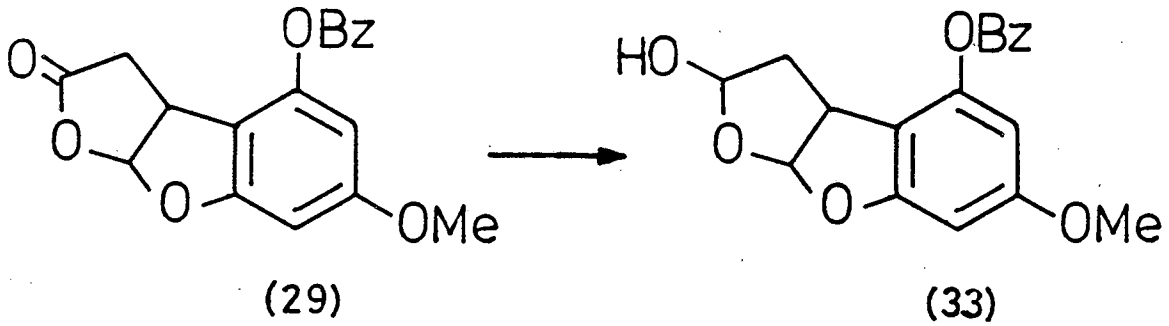
approach to the synthesis of MW206 held no distinct advantages over the existing synthesis,[4] and correspondingly, recourse to the latter was necessary.

2.4 SYNTHESIS OF MW206 (5).

The key step in the previously reported synthesis[4] of MW206 is the reductive rearrangement[9] of 5-benzyloxy-7-methoxy-4-formylcoumarin (28) to give the tricyclic lactone (29), which is further modified to produce the target compound. The aldehyde (28) was synthesised according to Buchi,[9] as follows.

The von Pechmann condensation[10] of phloroglucinol with ethyl acetoacetate in sulphuric acid afforded 5,7-dihydroxy-4-methylcoumarin (30) in 86% yield. There followed a selective monomethylation reaction which was described by Buchi,[9] but could not be repeated due to reasons of low solubility. For this step, the method of Roberts and Knight[11] was adopted, which involved the treatment of a heated solution of the dihydroxycoumarin (30) in sodium carbonate solution, with 1.2 equivalents of dimethyl sulphate. Filtration removed the unwanted dimethoxycoumarin, and acidification of the filtrates was followed by filtration, and subsequently recrystallisation from ethanol, to give pure 5-hydroxy-7-methoxy-4-methylcoumarin (31) in 23% yield.

The monomethoxy compound (31) was then benzylated in 78% yield by the use of benzyl chloride in refluxing acetone, in the presence of sodium iodide, with sodium carbonate as base.



(29) (33) (34) (35)

The suitably protected coumarin (32) was then converted to the 4-formyl compound (28) by oxidation with selenium dioxide in refluxing xylene. In order to verify the structure of this aldehyde with respect to the regiochemistry about the aromatic ring, a nuclear Overhauser experiment was carried out. No nuclear Overhauser enhancement (nOe) was observed between the methylene protons of the benzyl group and the aldehyde proton. The appearance of such an effect would have confirmed the structure as that which was required, but its absence does not necessarily imply the alternative structure, i.e. that with the benzyl and methyl protecting groups transposed. However, the structure was confirmed as 5-benzyloxy-7-methoxy-4-formylcoumarin by the appearance of nOe's to both aromatic protons upon irradiation of the methyl group, and only one nOe to an aromatic proton when the methylene group was irradiated.

The tricyclic lactone (29) was then prepared in 93% yield by the action of zinc in refluxing acetic acid on the formyl coumarin (28). This occurs by hydrogenation of the coumarin double bond, followed by rearrangement to form the lactone.

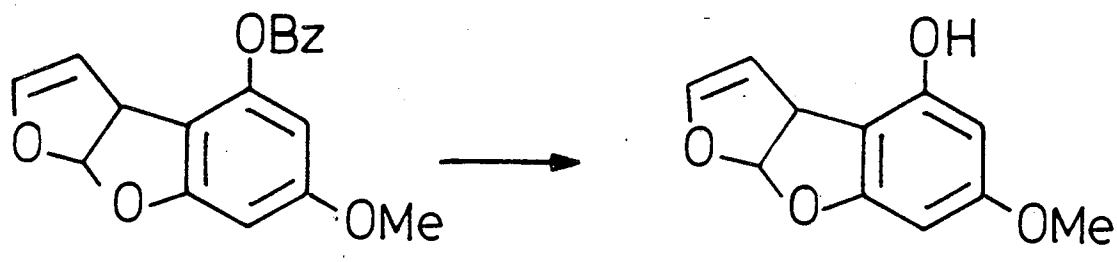
By the use of diisobutylaluminium hydride (DIBAL), it was possible to selectively reduce the lactone to give the hemiacetal (33).[4] This hydroxyl group was then acetylated quantitatively with acetic anhydride and sodium acetate in benzene, giving a diastereomeric mixture which was not resolved.

To introduce the terminal double bond, it was necessary to eliminate acetic acid, which could be achieved by pyrolysis. This was initially carried out using the flash vacuum pyrolysis technique described earlier. Subliming the tricyclic acetate (34) through a heated (500°C) silica tube at 0.001 mm Hg, gave a 30-50% yield of the desired elimination product (35). Yields decreased as the amount of starting material was increased, and the procedure was time-consuming as the diastereomeric mixture, a viscous oil, sublimed only slowly. Therefore, in order to obtain useful quantities of the elimination product, a method of pyrolysis more applicable to large scale was required.

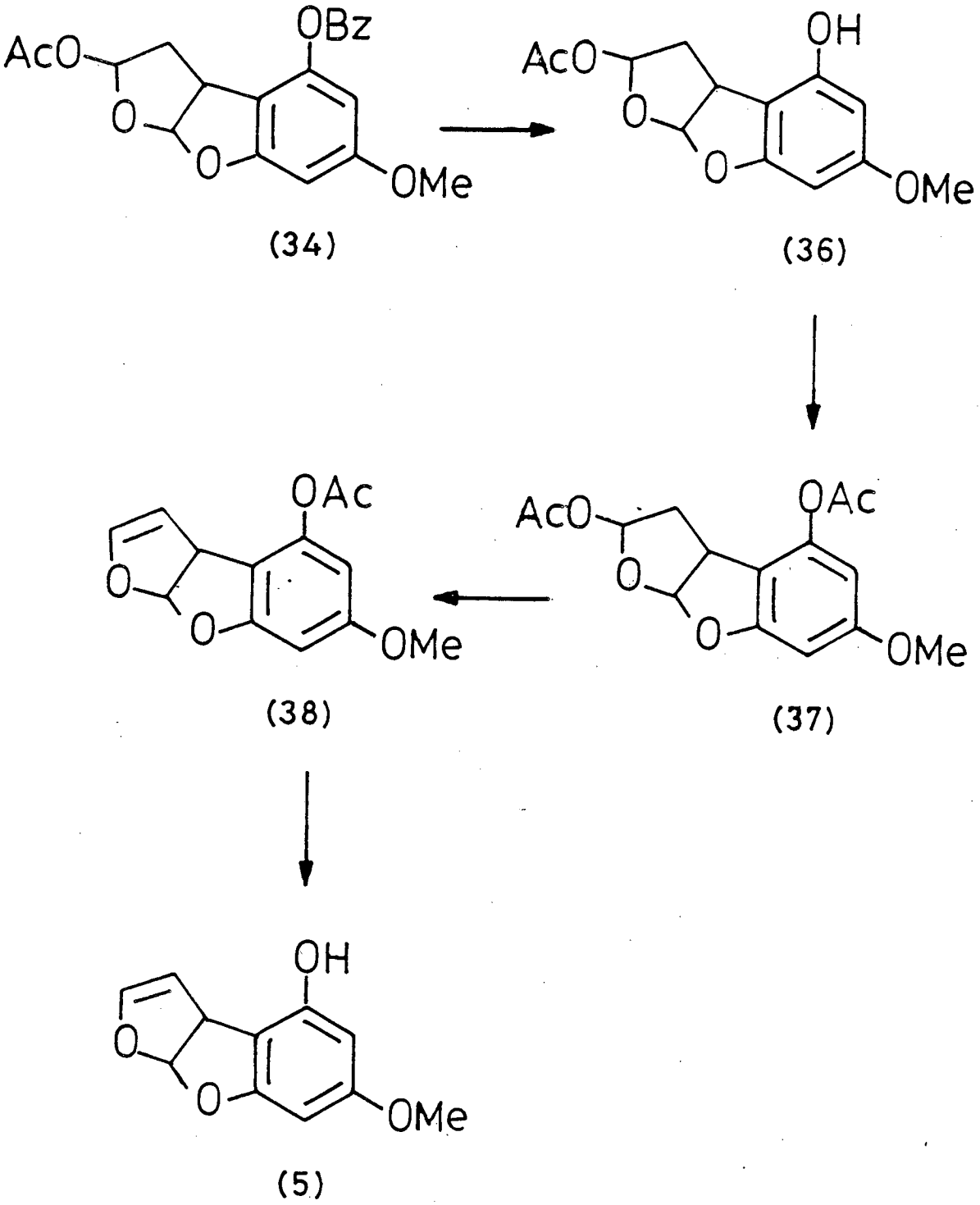
To this end, vertical flash vacuum pyrolysis was investigated. In this technique, the acetate was absorbed onto Celite and passed through a vertical silica column at 500°C and 0.01 mm Hg. Although this procedure could be used for relatively large quantities (ca. 500 mg), the yield (14%) was disappointing.

Refluxing the tricyclic acetate (34) in xylene or toluene-xylene mixtures was also unsuccessful, resulting only in decomposition products.

Finally, a technique was adopted[12] which allowed batches of a reasonable size to be processed, and gave yields comparable to flash vacuum pyrolysis. This involved slowly dropping a toluene solution of the acetate onto a vertical column of glass beads, heated by a furnace at 400°C, under a steady stream of nitrogen. The products were collected from the bottom of the column in a liquid



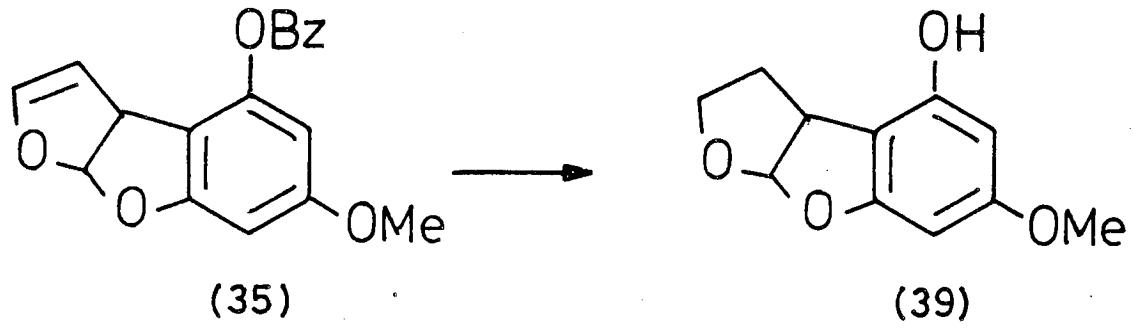
(35) (5)


AcO
OBz
O
O
OMe
(34)
AcO
OH
O
O
OMe
(36)
AcO
OAc
O
O
OMe
(37)
OAc
O
O
OMe
(38)
OH
O
O
OMe
(5)

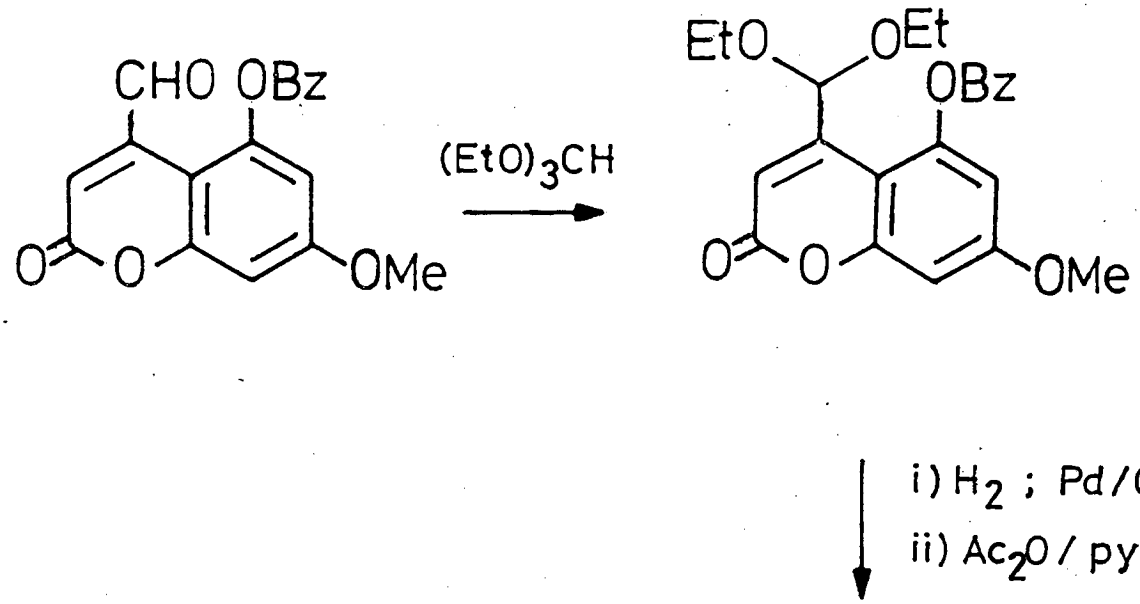
nitrogen trap and purified by preparative tlc.

The final deprotection step in the synthesis of MW206 i.e. the debenzylation of 4-benzyloxy-6-methoxy-3a,8a-dihydrofuro[2,3-b]benzofuran (35), could now be attempted. This was achieved, in good yield, by treatment with sodium in liquid ammonia. Accurate mass spectrometry of the product revealed that the parent ion at m/z 206 had the formula $C_{11}H_{10}O_4$, and the IR spectrum and melting point were also very similar to those previously reported[4] for the phenol (5). The proton nmr was also similar to that observed by Buchi, except that the aromatic protons were present as a pair of doublets at 6.10 and 5.93 ppm, rather than an AB multiplet at 5.88 ppm, as previously reported. The possibility of this compound being the isomer with the hydroxy and methoxy groups transposed was discounted however, in the light of the structural information from the nuclear Overhauser experiment described earlier, performed on the aldehyde (28). Further confirmation was provided when this compound co-chromatographed with the MW206 spot from an aflatoxin B_1 ammoniation mixture, on analytical tlc in several solvent systems.

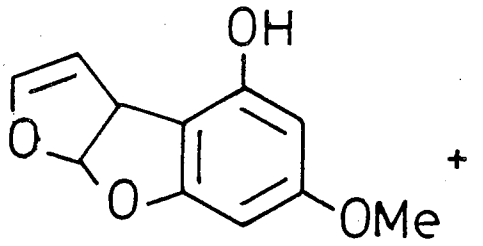
As a comparison, the method used by Buchi[4] to convert the tricyclic acetate (34) to MW206 was also carried out. The benzyl group was removed by hydrogenolysis of (34) in ethyl acetate using 10% palladium on carbon catalyst. The resultant phenol (36) was not isolated, but acetylated with acetic anhydride and sodium acetate in benzene to give the diacetate (37) in 71% overall yield from the monoacetate.



(35) (39)



Scheme (2)



(5)

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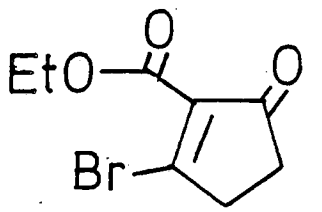
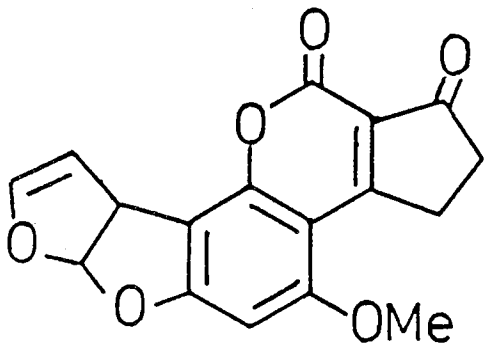
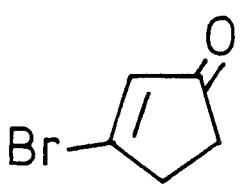


(40)



(1)



(41)

This compound was then pyrolysed in toluene solution on a column of glass beads at 400 °C, to give the acetate-elimination product (38) in 33% yield. Flash vacuum pyrolysis of the same compound at 500 °C and 0.03 mm Hg gave a yield of 32%.

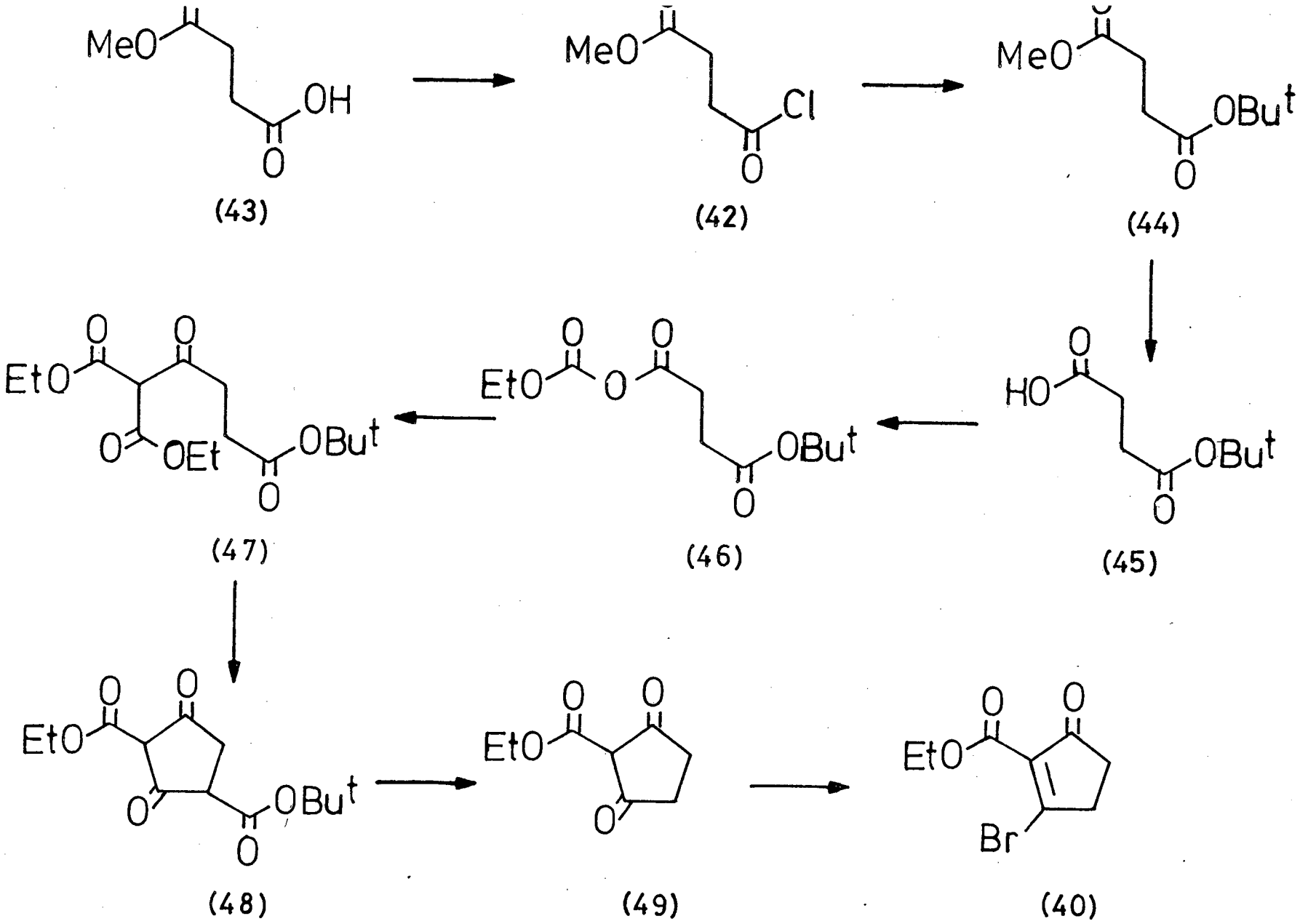
Saponification of the acetate (38) in methanolic potassium carbonate solution gave MW206 in quantitative yield.

Also, since it was available, 4-benzyloxy-6-methoxy-3a,8a-dihydrofuro[2,3-b]benzofuran (35) was hydrogenated to give, in 83% yield, 4-hydroxy-6-methoxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran (39), the dihydro-analogue of MW206. This is an alternative to the more concise synthesis of this compound previously documented by Roberts et al.,[11] outlined in scheme (2).

2.5 ATTEMPTED SYNTHESIS OF AFLATOXIN D_1.

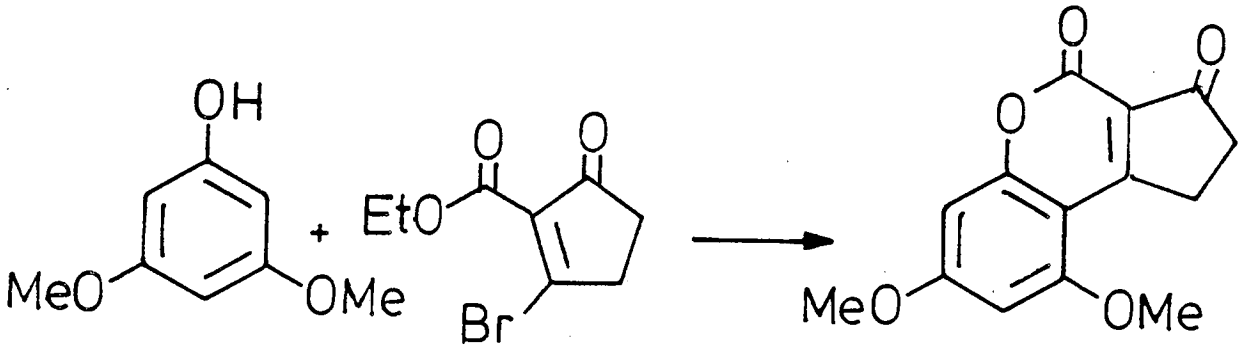
To complete the synthesis of aflatoxin D_1, it remained to attach a cyclopentenone ring at the 5-position of MW206. In Buchi's aflatoxin synthesis,[4] MW206 was subjected to a "modified von Pechmann reaction" where it was condensed with 2-carbethoxy-3-bromocyclopentenone (40), in the presence of zinc carbonate, to form aflatoxin B_1. The obvious analagous reaction using 3-bromocyclopentenone (41) was therefore attempted, but only starting materials were recovered.

Buchi found that the presence of zinc or magnesium carbonate was essential for this condensation, presumably due to the activation of the bromide via chelation of the

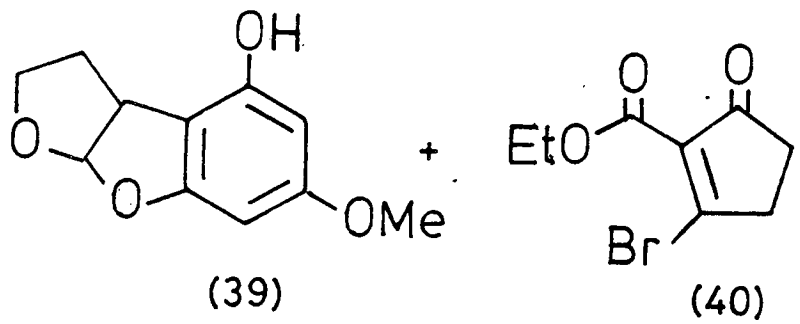

MeO
OH
O
(43)
MeO
Cl
O
(42)
MeO
OBut
O
(44)
HO
OBut
O
(45)
EtO
O
OBut
O
(46)
EtO
O
OEt
OBut
O
(47)
EtO
O
OBut
O
(48)
EtO
O
(49)
EtO
Br
(40)

metal ion to the carbonyl groups. The absence of any such chelation when using 3-bromocyclopentenone explains the lack of reaction, and if the first step in the condensation to produce aflatoxin B_1 is esterification at the phenol, then this type of approach would not be of any use. Therefore, 2-carbethoxy-3-bromocyclopentenone (40) was synthesised, according to Buchi,[13] in order to investigate this possibility.

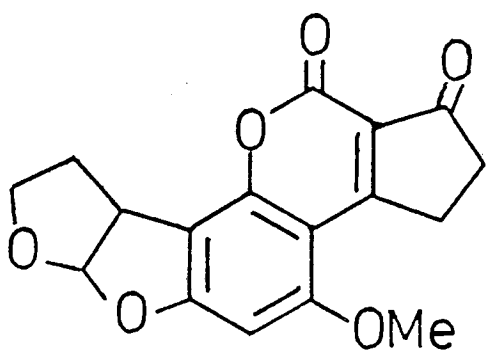
Firstly, methyl succinyl chloride (42) was prepared by the action of thionyl chloride on methyl hydrogen succinate (43).[14] The acid chloride (42) was reacted with t-butanol in N,N-dimethylaniline to produce t-butyl methyl succinate (44). Steric hindrance by the t-butyl group allowed the compound (44) to be selectively hydrolysed, giving t-butyl hydrogen succinate (45) on treatment with a mixture of aqueous sodium hydroxide and 1,4-dioxan. The mixed anhydride (46) formed by the addition of ethyl chloroformate to a solution of t-butyl hydrogen succinate (45) and triethylamine in toluene, was reacted with an ethereal solution of diethylethoxymagnesiomalonate affording, after acidification, diethyl 3-carbo-t-butoxypropionylmalonate (47). This compound was cyclised using potassium t-butoxide in refluxing benzene, to form the substituted cyclopentanedione (48). Hydrolysis of the t-butyl ester, followed by decarboxylation to give 2-carbethoxycyclopentane-1,3-dione (49) was brought about using p-toluenesulphonic acid in refluxing benzene. Finally, the dione (49) was converted to the bromide (40) by treatment with oxalyl bromide.[4]



(50) (40) (51)



(39) (40)



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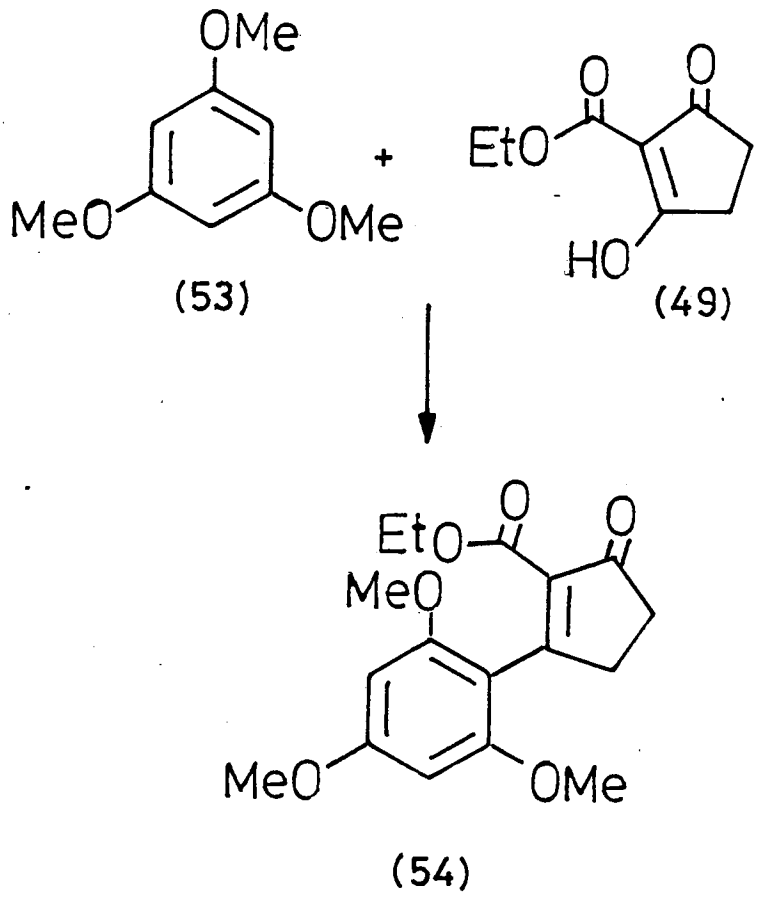
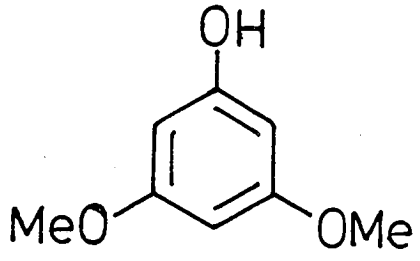


(53) (49)

(54)



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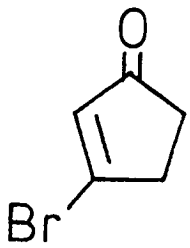

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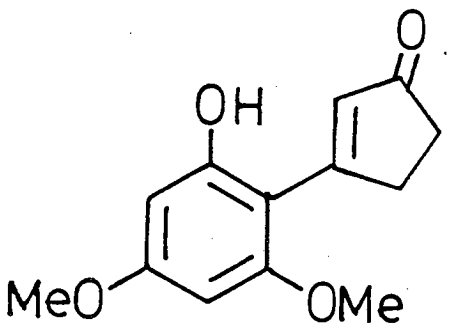

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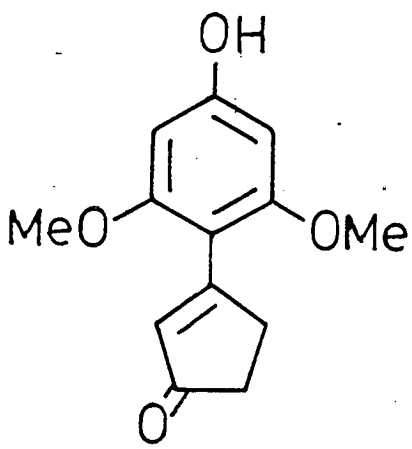

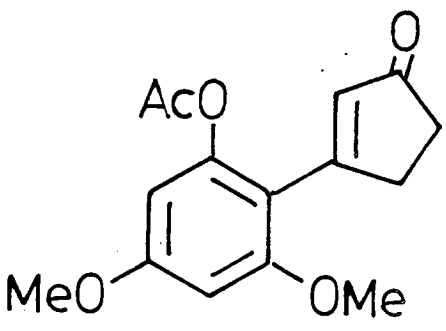

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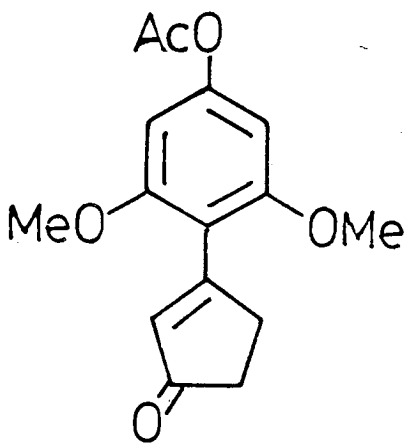


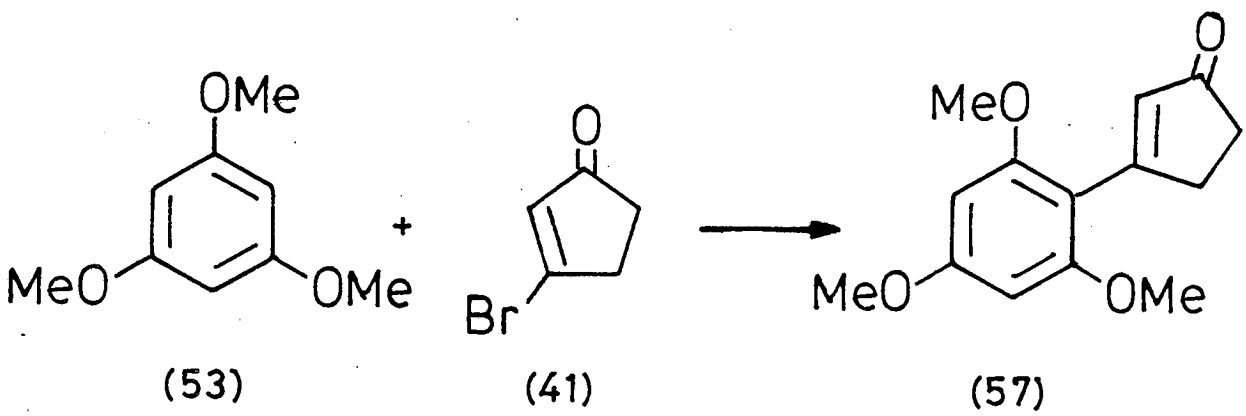
(56)

Having prepared the bromide (40), an example of Buchi's condensation reaction was investigated by refluxing it with 3,5-dimethoxyphenol (50) in methylene chloride, in the presence of zinc carbonate. The expected coumarin (51) was produced in 33% yield. Additionally, using phenol (39) in place of 3,5-dimethoxyphenol, it was possible to synthesise (±)-aflatoxin B_2 (52).

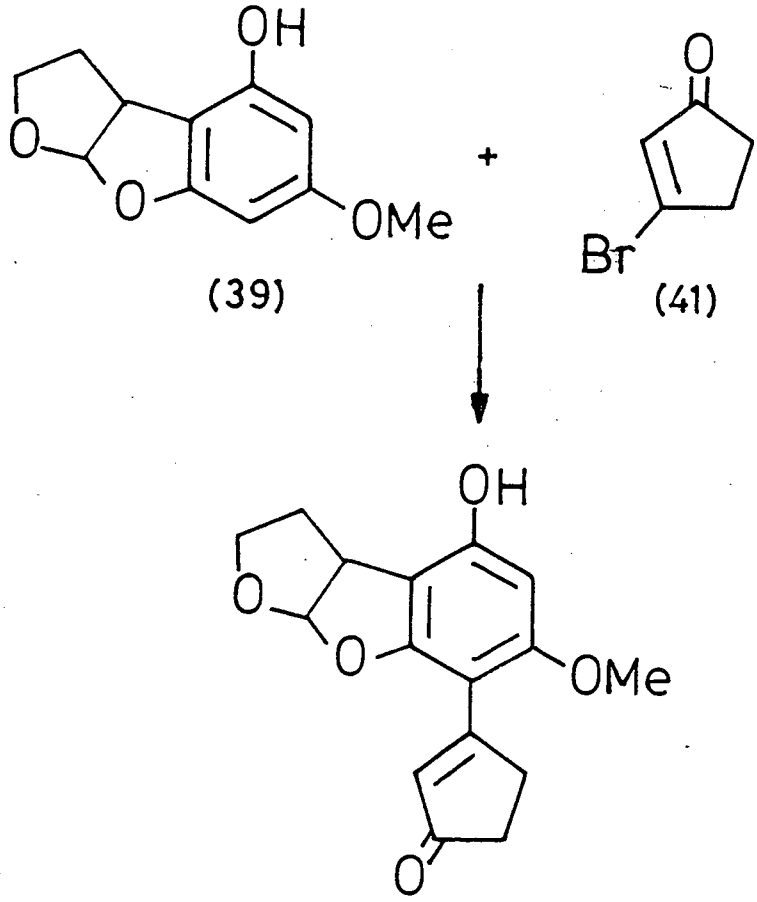
However, the lack of any reaction with the bromide (40) when using 1,3,5-trimethoxybenzene (53) in place of a phenol suggests that formation of the ester of the phenol might indeed be the initial step in the zinc carbonate catalysed condensation reaction. Alternatively, the presence of the phenolic group might be required to activate the ring sufficiently for the bromide displacement step.

It was possible to attach the five-membered ring to the benzenoid nucleus when 1,3,5-trimethoxybenzene was reacted with the dione (49) in the presence of phosphorus pentoxide. However, the yield of the product (54) was low (7%) and moreover, Buchi has demonstrated[4] that the dihydrofurobenzofuran system is not stable under such conditions, so that this method could not be applied to aflatoxin synthesis.

In an alternative approach, Lewis acid catalysis was considered as a means of attaching the cyclopentenone ring in model systems. The boron trifluoride etherate catalysed reaction of 3,5-dimethoxyphenol and 3-bromocyclopentenone (41) gave a polar product that was shown by nmr to be a mixture of two components. Acetylation of this mixture gave



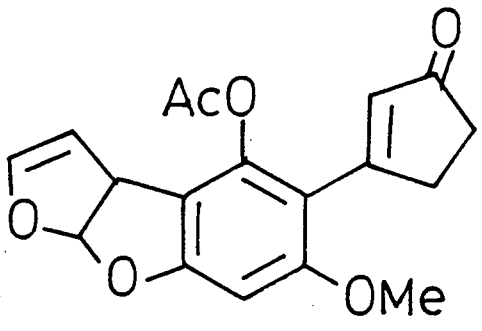
(53) (41) (57)



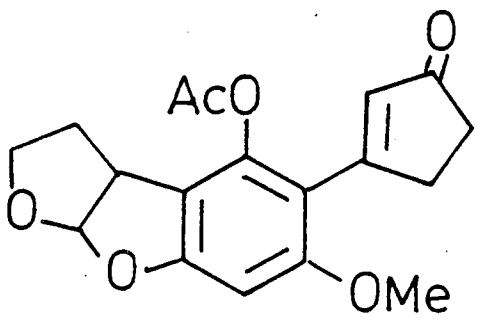
(39) (41)

(59)

(60)



(4)



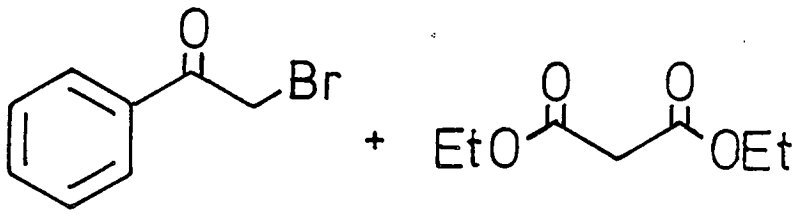
(58)

a product which exhibited two distinct acetate signals of approximate ratio 1:1. The aromatic region confirmed that these were due to substitution occurring either in the ortho position or the para position, to give (55) or (56). It appears that para substitution is favoured, since if ortho and para substitution were equally likely, a ratio of 2:1 would have been observed for the acetate signals. Unfortunately, no solvent system could be found to separate either the original mixture, or the acetates.

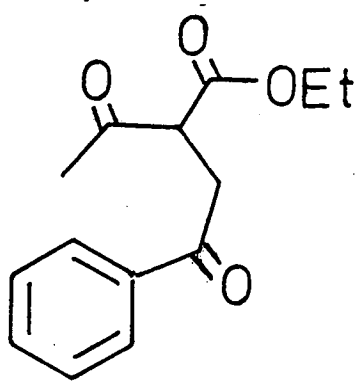
In order to obtain a single isomer of a model compound, 1,3,5-trimethoxybenzene was treated with 3-bromocyclopentenone in boron trifluoride etherate. The desired product (57) was produced in 52% yield.

To synthesise aflatoxin D_1, the boron trifluoride catalysed reaction of MW206 with 3-bromocyclopentenone was attempted, but resulted, as expected, only in the decomposition of the former.

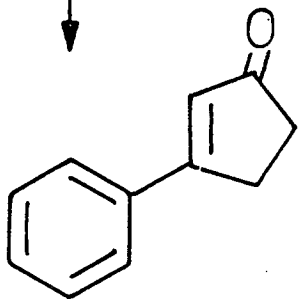
The dihydro analogue (39) of MW206 however, was found to react in such a procedure, albeit in small yield, to give only one isolable product. This phenol was acetylated to facilitate nmr analysis. However, the resonances due to the olefinic proton and the two methylene groups of the cyclopentenone portion of the moleclule differed markedly from those of aflatoxin D_1 acetate (4), prepared from aflatoxin D_1 produced by the ammoniation of aflatoxin B_1. Therefore, to carry the comparison further, aflatoxin D_1 acetate was hydrogenated, to form the dihydro- derivative (58). Again, the cyclopentenone resonances were different,



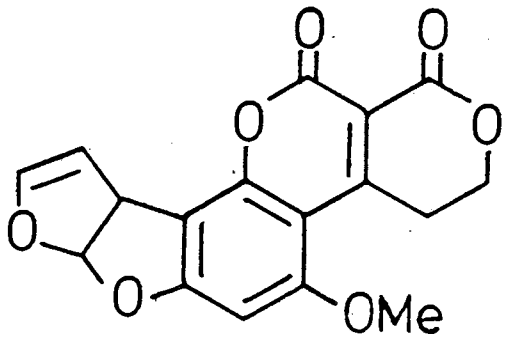
(62)



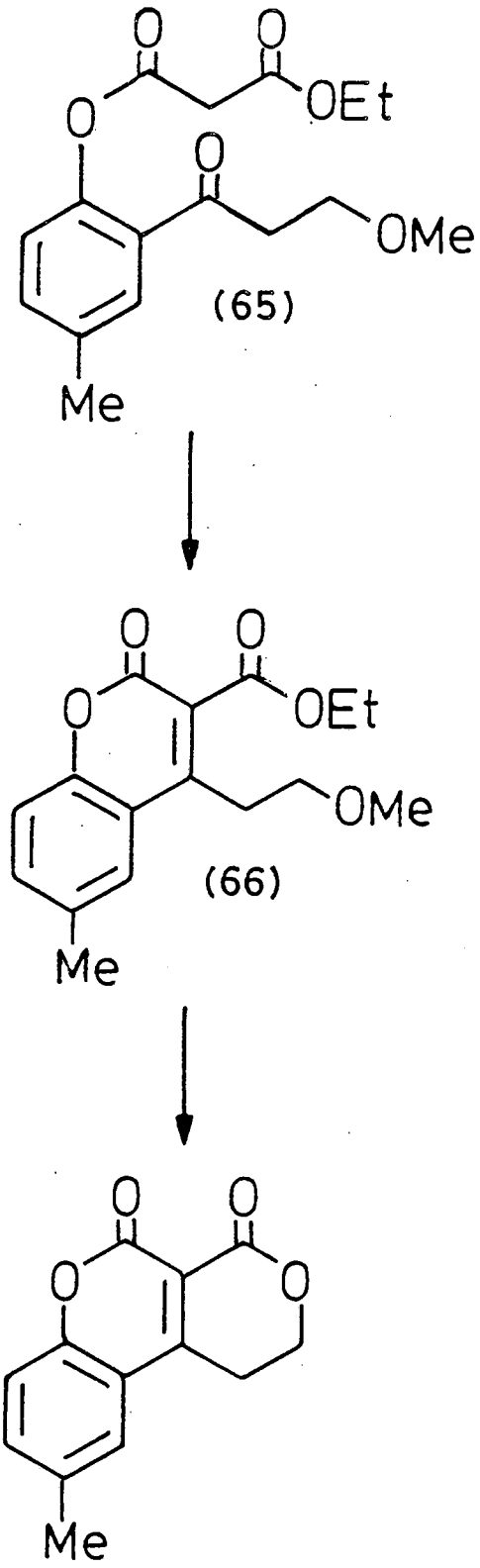
(63)



(61)



(64)

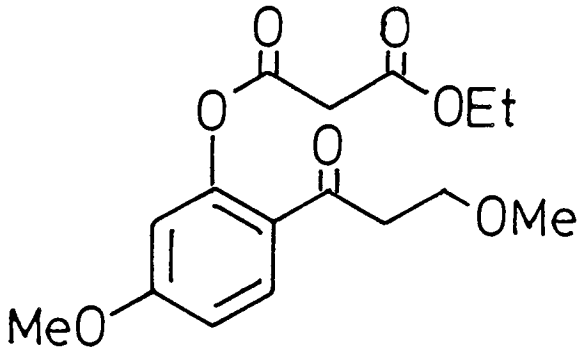

O
O
OEt
O
O
OMe
Me
(65)
O
O
OEt
O
OMe
Me
(66)
O
O
O
O
Me

suggesting that the disposition of the cyclopentenone fragment in the synthetic material is para-, rather than ortho- to the phenol. This view was confirmed, following a nuclear Overhauser experiment on the synthetic acetate. An nOe of 1% was observed to the aromatic proton, on irradiation of the acetate methyl, which would not be possible if they were situated para- to one another. Thus, the synthetic phenol has structure (59), which gives acetate (60) on acetylation.

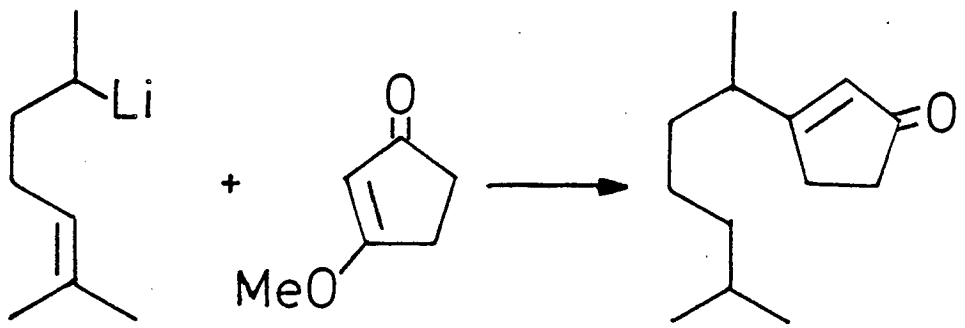
Although the Lewis acid approach has provided a route to model compounds, it is evidently not suitable for the complete synthesis of aflatoxin D_1, so an alternative was sought.

The literature[15] shows that 3-phenylcyclopentenone (61) can be synthesised by the action of the sodium salt of ethyl acetoacetate on bromoacetophenone (62), presumably first giving the adduct (63), which on treatment with base, cyclises and decarboxyethylates to the desired product (61).

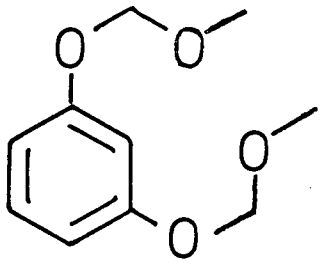
The above reaction might appear to offer a route to aflatoxin D_1 and other models, but the findings of Roberts et al.[16] throw this into doubt. These authors were investigating ways of building up the coumarin-lactone system of aflatoxin G_1 (64). Base induced cyclisation of the model compound (65) was found to proceed effectively, giving the coumarin (66), which could be further cyclised using sulphuric acid to elaborate the other lactone ring. However, the model compound (67) resisted similar attempts



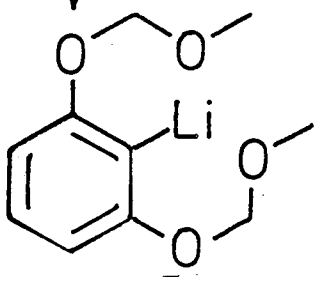
(67)

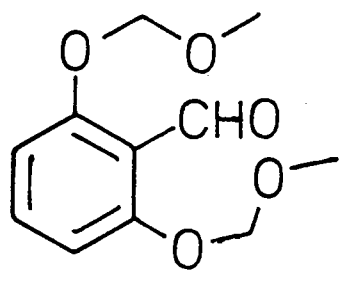


(69) (68)

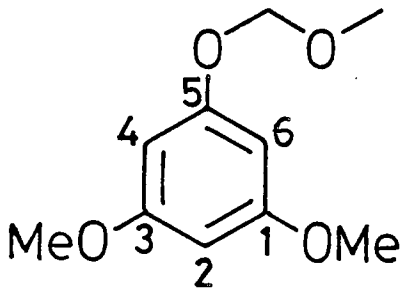


(70)





(71)



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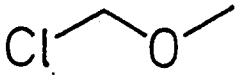
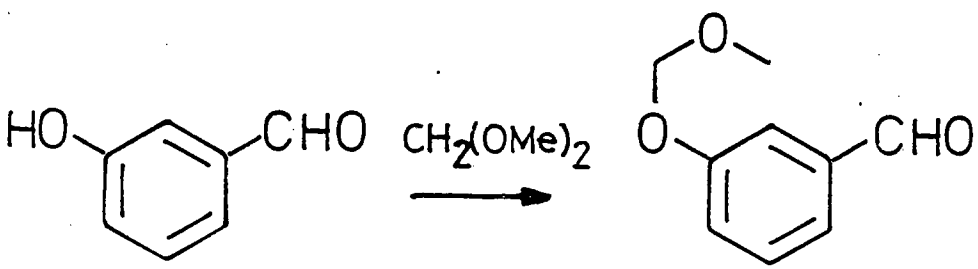


(73)



(74)

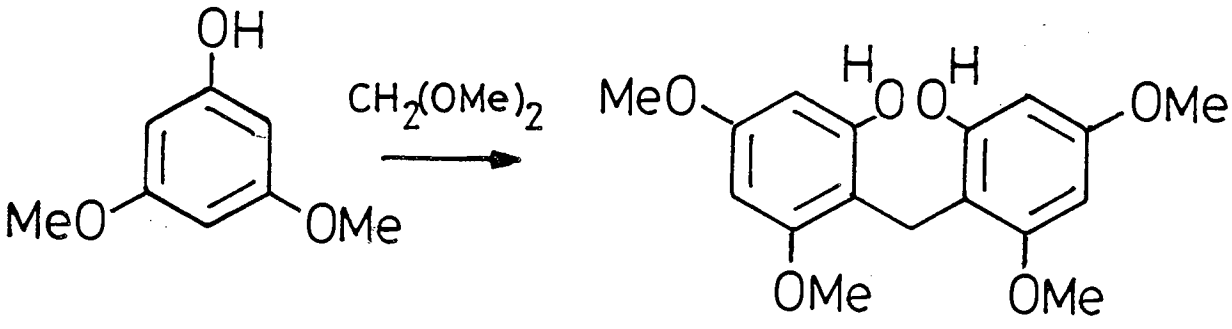
to cyclise it. This lack of reactivity was attributed to deactivation of the ketone carbonyl towards nucleophilic attack, due to electron release from the para- methoxy group. Such a deactivation would probably also occur with the required methoxy analogues of bromoacetophenone, and therefore this reaction was not investigated.

It has been shown by Corey[17] that 3-methoxycyclopentenone (68) will react with 2-(6-methyl-5-heptenyl)-lithium (69) at -78 °C, to give addition of the cyclopentenone fragment, with loss of the methoxyl group.

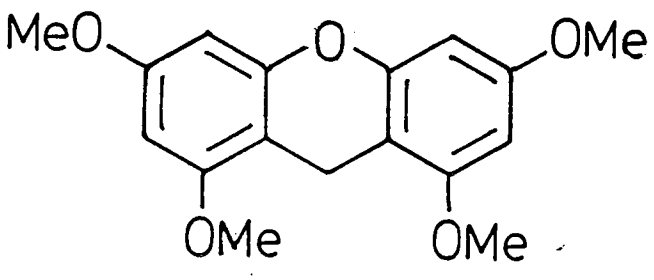
In addition, lithium salts can be formed on aromatic rings which are activated by electon releasing substituents, such as methoxyl. For example,[18] 1,3-bis(O-methoxymethyl)resorcinol (70) has been treated with n-butyllithium to give lithiation in the 2- position, and followed by the addition of DMF, to form the benzaldehyde (71). It appeared that such an approach would now be the most likely to succeed, and work was commenced with model compounds.

The model chosen was 1,3-dimethoxy-5-(O-methoxymethyl)benzene (72), since the methoxymethyl function is known to give extra stabilisation of the aromatic lithium compound,[19] hence favouring substitution at the 4- position, and also because it is a protecting group that can be easily removed under mild conditions.[20]

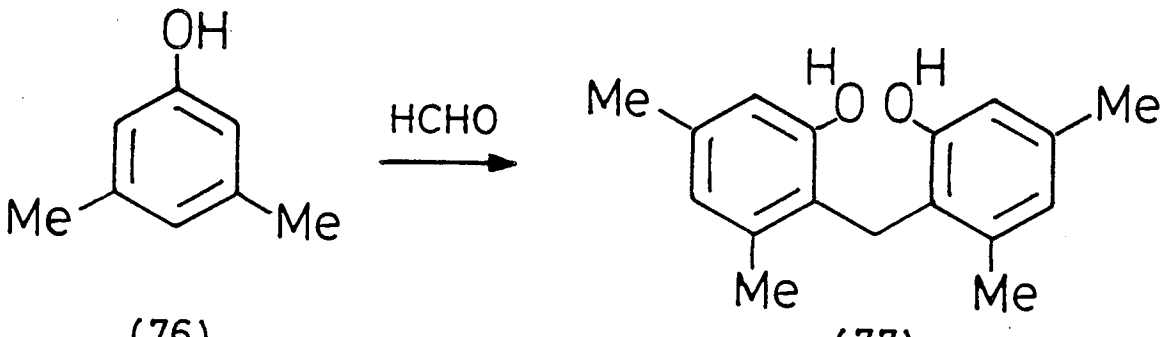
The desired compound was to be formed from 3,5-dimethoxyphenol (50), and in order to avoid the use of the extremely hazardous chloromethylmethyl ether (73) required


OH
MeO
OMe
$CH_2(OMe)_2$
H
O
O
H
MeO
OMe
OMe
OMe

(75)


MeO
O
OMe
OMe
OMe

(78)


OH
Me
Me
HCHO
H
O
O
H
Me
Me
Me
Me

(76)

(77)

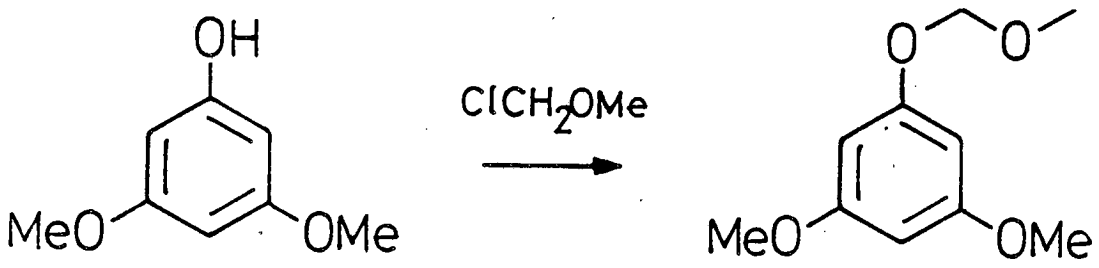
in the standard preparation of methoxymethyl ethers, an alternative method described by Yardley and Fletcher[21] was tried initially. These workers have introduced the methoxymethyl group to a number of phenols, e.g. 3-hydroxybenzaldehyde (74), by treatment with dimethoxymethane using p-toluenesulphonic acid catalysis in refluxing methylene chloride.

Using 3,5-dimethoxyphenol in this reaction gave one major product, but it was evident that this did not contain the methoxymethyl function. On consideration of the proton nmr and mass spectra of the product, it appeared to have structure (75), formed by reaction of one equivalent of dimethoxymethane with two of the phenol. Such behaviour might be anticipated, since dimethoxymethane is considered as a formaldehyde equivalent, and m-xylenol (76) has been shown to condense with formaldehyde to form the analagous product (77).[22]

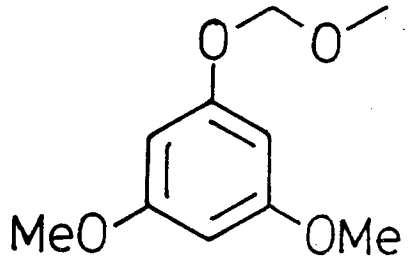
The structure of (75) was later confirmed by suspending the compound in cold concentrated sulphuric acid for 2 days. After workup, the corresponding tetramethoxy[9H]xanthene (78) was isolated. This formally represents a new synthetic route to xanthenes and xanthones.

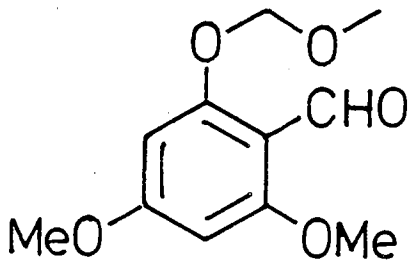
The fact that 3,5-dimethoxyphenol is more activated towards electrophilic aromatic substitution than the phenols used by Yardley and Fletcher probably explains the observed course of reaction.

Therefore, 1,3-dimethoxy-5-(O-methoxymethyl)benzene



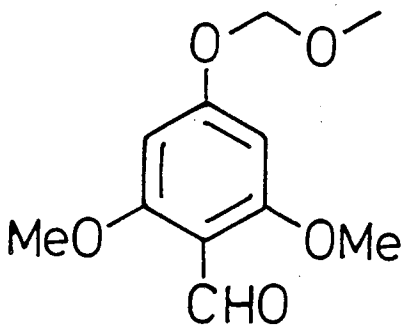
(72)


O
O
MeO
OMe

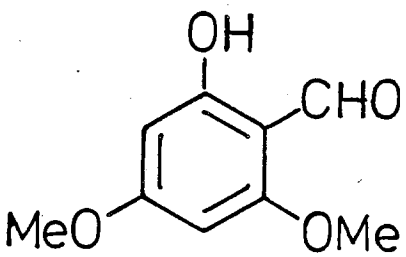

O
O
CHO
MeO
OMe

(80)

+

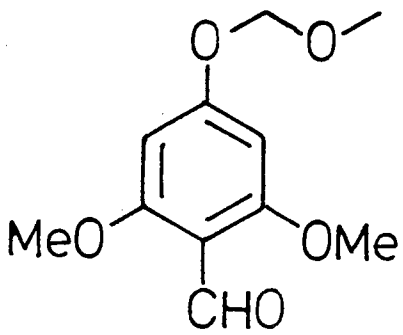

O
O
MeO
OMe
CHO

(79)


OH
CHO
MeO
OMe

(81)

+


O
O
MeO
OMe
CHO

(79)

(72) was prepared using the standard route,[18] where the phenoxide anion formed by treatment of the phenol (50) with sodium hydride in DMF, was reacted with chloromethylmethyl ether, displacing the chlorine, thus forming the methoxymethyl ether in 95% yield.

Next, it required to be demonstrated that lithiation could indeed be effected on the aromatic ring. Hence, the procedure used by Townsend,[18] described earlier, to produce the benzaldehyde (71) was investigated, using methoxymethyl ether (72).

After several attempts it became apparent that particular care was required to exclude atmospheric moisture from the apparatus, in order to generate the lithiated intermediate. Excess n-butyllithium in hexane was added to a solution of the aromatic ether (72) in dry THF. The solution was refluxed for one hour, to form the lithiated aromatic compound, then quenched with dry DMF. Analytical tlc showed the presence of a single spot at a lower R_f than the starting material. This product was isolated by preparative tlc and analysed by proton nmr spectroscopy which showed that it comprised a mixture of the two possible isomeric benzaldehydes. Apart from two aldehyde signals being visible, the aromatic region consisted of a singlet, due to the symmetrical isomer (79), and a pair of meta- coupled doublets, due to the unsymmetrical isomer (80). Integration of the signals showed that the symmetrical and unsymmetrical isomers were present in a ratio of 1:2.6 respectively. This ratio shows

that lithiation is favoured slightly more in a position ortho- to the methoxymethyl ether, or alternatively, para- to the methyl ether.

The mixture of benzaldehydes was inseparable, but it was thought that on deprotection the two isomers could be isolated, since the o-hydroxybenzaldehyde (81) would be less polar due to intramolecular hydrogen bonding. After mild acid hydrolysis, the o-hydroxybenzaldehyde (81) was isolated, and surprisingly, the p-formyl compound (79) was recovered, with the methoxymethyl protection intact.

Although this result was encouraging, repeated attempts to produce an aflatoxin D_1 analogue by replacing the DMF in the above reaction with either 3-bromocyclopentenone or 3-methoxycyclopentenone, at temperatures from -78 °C to 20 °C, all met with failure.

2.6 GENERAL EXPERIMENTAL CONDITIONS AND PROCEDURES.

Melting points were determined using a Reichert hot stage microscope and are uncorrected. Analyses were determined on a Carlo Erba 1106 elemental analyser. Infra-red spectra were obtained on a Perkin-Elmer 781 spectrophotometer. Ultra-violet spectra were determined on a Pye Unicam SP8-400 spectrophotometer. Proton nmr spectra were obtained on Varian EM360, Bruker WP80SY, WP200SY, and WH360 instruments. All quoted chemical shifts are relative to tetramethyl silane, δ_H = 0.00 ppm. Mass spectra were obtained on Kratos MS 902 and MS 50 TC spectrometers. Thin layer chromatography was carried out on either analytical (5 x 20 cm) or preparative (20 x 20 cm) glass plates, coated with a 0.5 mm layer of silica gel (Fluka AG 60765 Kieselgel GF_{254}). Chromatograms were visualised under ultra-violet light (254 nm), by development in an iodine tank, or by spraying with a 1% solution of Fast Blue B in water.

2.7 EXPERIMENTAL.

Isolation of Aflatoxin B_1 (1) and Aflatoxin G_1 (64).

Aspergillus flavus CMI 120920 spores were maintained under liquid paraffin on Czapek-Dox agar slopes. The slopes were used to inoculate three 500 ml Erlenmeyer flasks containing "low salts" medium,[23] and grown for 3 d., on a rotary shaker at 26 °C. This inoculum was then used to inoculate 2 l of "low salts" medium distributed between 14 x 500 ml Erlenmeyer flasks, and incubated for 7 d., at 26 °C, on a rotary shaker. The contents of the culture flasks were

filtered, and the liquors were reserved. The mycelium was homogenised with acetone in a Waring blender, then filtered. The process was repeated twice on the residual mycelium, and the combined acetone extracts were concentrated in vacuo, leaving an orange, aqueous solution. This solution was extracted with chloroform (3 x), as was the previously reserved liquors layer, and the extracts were combined, then concentrated in vacuo. The residual solid was dissolved in a mixture of methanol and water (9:1), and washed with (40-60) petroleum ether (2 x). The polar layer was then concentrated in vacuo, to an aqueous solution, which was extracted with chloroform (3 x). The chloroform layer was dried ($MgSO_4$), and concentrated in vacuo, to give the crude extract (389 mg). The crude extract was purified by preparative tlc (71% chloroform - 12% acetone - 17% (40-60) petroleum ether), and the major blue fluorescent band gave aflatoxin B_1 (1) (125 mg; 62 mg/l). δ_H (80 MHz; $CDCl_3$) 2.62 (2H, m, H-2), 3.38 (2H, m, H-3), 3.93 (3H, s, OCH_3), 4.74 (1H, dt, J 7, 2 Hz, H-9a), 5.45 (1H, t, J 2 Hz, H-9), 6.38 (1H, s, Ar-H), 6.40 (1H, dd, J 3, 2 Hz, H-8), 6.79 (1H, d, J 7 Hz, H-6a) ppm. The major green fluorescent band gave aflatoxin G_1 (64) (70 mg; 35 mg/l). δ_H (80 MHz; $CDCl_3$) 3.42 (2H, t, J 6 Hz, H-4), 3.92 (3H, s, OCH_3), 4.40 (2H, t, J 6 Hz, H-3), 4.75 (1H, dt, J 7, 2 Hz, H-10a), 5.45 (1H, t, J 2 Hz, H-10), 6.40 (1H, s, Ar-H), 6.45 (1H, dd, J 3, 2 Hz, H-9), 6.79 (1H, d, J 7 Hz, H-7a) ppm.

Initial Ammoniation of Aflatoxin B_1 (1).

Concentrated ammonium hydroxide (s.g. 0.88; 10 ml) was

added to aflatoxin B_1 (1) (20 mg, 0.06 mmol), and the mixture was heated at reflux overnight. The solution was then neutralised by the addition of 2 M HCl, then extracted with ethyl acetate. The organic layer was then dried ($MgSO_4$), and concentrated in vacuo to a solid. Analytical tlc of the product (2% methanol - 98% chloroform) revealed aflatoxin B_1 at R_f 0.33, and a non-fluorescent spot at R_f 0.26. The non-fluorescent material was separated by preparative tlc (2% methanol - 98% chloroform, then 10% methanol - 90% chloroform) to give aflatoxin D_1 (2) (6 mg, 0.02 mmol; 33%).

Treatment of Sterigmatocystin (3) with Ammonium Hydroxide.

A mixture of sterigmatocystin (3) (52 mg, 0.16 mmol) and concentrated ammonium hydroxide solution (s.g. 0.88; 10 ml) was heated at reflux overnight. The solution was then neutralised by the addition of 2 M HCl, then extracted with ethyl acetate. The organic layer was dried ($MgSO_4$), then concentrated in vacuo, to an orange solid. The solid was purified by preparative tlc (5% methanol - 95% chloroform). The orange band gave unreacted sterigmatocystin (3) (50 mg, 0.15 mmol; 96%).

Typical Reaction of Aflatoxin B_1 (1) with Ammonium Hydroxide Solution.

To aflatoxin B_1 (76 mg, 0.24 mmol) in a round-bottomed flask was added concentrated ammonium hydroxide solution (s.g. 0.88; 20 ml), and the flask was sealed and placed in an oven at 50 °C for 21 d. The contents of the flask were

then lyophilised, and separated by preparative tlc (7% acetone - 93% methylene chloride). The lower non-fluorescent band gave aflatoxin D_1 (2) (40 mg, 0.14 mmol, 57%). δ_H (200 MHz; $CDCl_3$-CD_3OD) 2.52 (2H, m, H-5), 3.22 (2H, m, H-4), 3.81 (3H, s, OCH_3), 4.67 (1H, dt, J 7.1, 2.3 Hz, H-3a'), 5.43 (1H, t, J 2.4 Hz, H-3'), 6.20 (1H, s, Ar-H), 6.48 (1H, t, 1.7 Hz, H-2), 6.50 (1H, dd, J 2.7, 2.1 Hz, H-2'), 6.73 (1H, d, J 7.2 Hz, H-8a') ppm.

The blue fluorescent band gave residual aflatoxin B_1 (1) (11.3 mg, 0.04 mmol, 15%), and the upper non-fluorescent band gave MW206 (5) (4.7 mg, 0.02 mmol, 9%). δ_H (80 MHz, $CDCl_3$) 3.72 (3H, s, OCH_3), 4.55 (1H, dt, J 7, 2 Hz, H-3a), 5.32 (1H, t, J 2 Hz, H-3), 5.91 (1H, d, J 2 Hz, Ar-H), 6.11 (1H, d, J 2 Hz, Ar-H), 6.43 (1H, dd, J 3, 2 Hz, H-2), 6.67 (1H, d, J 7 Hz, H-8a) ppm.

Aflatoxin D_1 acetate (4).

Aflatoxin B_1 (1) (26 mg, 0.08 mmol) was transferred to a round-bottomed flask, and concentrated ammonium hydroxide solution (s.g. 0.88; 10 ml) was added. The flask was stoppered and sealed, then placed in an oven at 56 °C for 14 days. The contents were then lyophilised, and the product was subjected to preparative tlc (10% methanol - 90% methylene chloride), the non-fluorescent at R_f 0.47 giving aflatoxin D_1 (2) (10.2 mg, 0.04 mmol, 43%). A sample of this product was derivitised by heating for 1 h., at 110 °C, with pyridine (1 ml) and acetic anhydride (1 ml). The solution was then poured onto ice and extracted with ethyl acetate. The organic layer was dried ($MgSO_4$) and

concentrated in vacuo to a solid which was purified by preparative tlc (4% acetone - 96% chloroform), the blue fluorescent band giving aflatoxin D_1 acetate (4). δ_H (80 MHz; $CDCl_3$) 2.22 (3H, s, O_2CCH_3), 2.46 (2H, m, H-5), 2.99 (2H, m, H-4), 3.79 (3H, s, OCH_3), 4.47 (1H, dt, J 7, 2 Hz, H-3a'), 5.15 (1H, t, J 2 Hz, H-3'), 6.15 (1H, m, H-2), 6.46 (2H, m, H-7' and H-2'), 6.70 (1H, d, J 7 Hz, H-8a') ppm.

Aflatoxin D_1 acetate (4).

Aflatoxin B_1 (1) (52 mg, 0.17 mmol) and concentrated ammonium hydroxide solution (s.g. 0.88; 20 ml) were placed in a flask, which was sealed and heated at 58 °C for 21 d. The product was lyophilised, then dissolved in water (50 ml). The solution was acidified (2 M HCl), then extracted with ethyl acetate. After drying ($MgSO_4$), and concentration in vacuo, the organic layer yielded a solid, which was heated at 100 °C for 75 min., with pyridine (2 ml) and acetic anhydride (1 ml). The solution was then poured onto a mixture of ice (20 g) and 2 M hydrochloric acid (20 ml), then extracted with chloroform. The chloroform extract was washed with water, then dried ($MgSO_4$), and concentrated in vacuo, to a solid. This solid was then subjected to preparative tlc (10% acetone - 90% chloroform). The blue fluorescent band was removed, and further purified by repeated preparative tlc (5% acetone - 95% methylene chloride), giving aflatoxin D_1 acetate (4) (20 mg, 0.06 mmol, 36%), which gave fine needles from ethanol, m.p. 158-160 °C (lit.[1] m.p. 155-157 °C), (M^+: 328.09464. $C_{18}H_{16}O_6$ requires 328.09469); ν max ($CHCl_3$) 1750, 1700, 1630, 1600

cm^{-1}; λ max (MeOH) 227, 288, 316 nm (ϵ 12600, 4700, 6800); δ_H (200 MHz; $CDCl_3$) 2.22 (3H, s, O_2CCH_3), 2.46 (2H, m, H-5), 2.97 (2H, m, H-4), 3.79 (3H, s, OCH_3), 4.47 (1H, dt, J 7.1, 2.2 Hz, H-3a'), 5.14 (1H, t, J 2.5 Hz, H-3'), 6.15 (1H, t, J 1.8 Hz, H-2), 6.45 (1H, dd, J 2.7, 2.0 Hz, H-2'), 6.46 (1H, s, H-7'), 6.69 (1H, d, J 7.1 Hz, H-8a') ppm.

3,4-Dihydroxytetrahydrofuran (11).

Meso-erythritol (10) (100 g, 0.82 mol) and Amberlite acidic ion-exchange resin (10 g) were placed in a distillation flask and heated (oil bath; 140 °C) until the erythritol melted. The mixture was then distilled in vacuo (132 °C; 2 mm Hg) to give a clear liquid (63 g). This liquid was redistilled (86 °C; 0.5 mm Hg) to give 3,4-dihydroxytetrahydrofuran (11) (49.5 g, 0.48 mol; 58%).

cis-3-Acetoxy-4-hydroxytetrahydrofuran (12).

3,4-Dihydroxytetrahydrofuran (11) (42.8 g, 0.412 mol) was dissolved in dry tetrahydrofuran (200 ml). Triethyl orthoacetate (100 ml, 0.616 mol) and trifluoroacetic acid (0.4 ml) were also added, and the solution was heated at reflux, under an atmosphere of nitrogen, for 44 h. After cooling, the solvent was removed in vacuo and the residual oil was added to a mixture of acetone (145 ml) and 5% aqueous oxalic acid (14.5 ml), and stirred at room temperature for 30 min. Anhydrous sodium sulphate (5 g) and potassium bicarbonate (1 g) were added, and stirring was continued for 15 min. The mixture was then filtered, and the solvent removed in vacuo to leave a yellow oil which was purified by distillation (80 °C; 0.7 mm Hg) to give

cis-3-acetoxy-4-hydroxytetrahydrofuran (12) (44.8 g, 0.307 mol; 75%) as a colourless liquid. δ_H (200 MHz, $CDCl_3$) 2.10 (3H, s, O_2CCH_3), 2.55 (1H, br, OH), 3.66 (1H, dd, J 9,5 Hz, H-5), 3.79 (1H, dd, J 10,4 Hz, H-2), 3.93 (1H, dd, J 9,6 Hz, H-5), 4.02 (1H, dd, J 9,6 Hz, H-2), 4.41 (1H, q, J 6 Hz, H-4), 5.08 (1H, q, J 6 Hz, H-3) ppm.

4-Acetoxytetrahydrofuran-3-one (13).

cis-3-Acetoxy-4-hydroxytetrahydrofuran (12) (8.05 g, 55 mmol) was dissolved in methylene chloride (15 ml) and added dropwise to a suspension of pyridinium chlorochromate (23.8 g, 110 mmol) and Celite (5.9 g) in methylene chloride (150 ml). The slurry was stirred mechanically under an atmosphere of argon for 40 h, then filtered, washed with methylene chloride and the filtrates were concentrated in vacuo to a dark oil. Ether (100 ml) was added to the oil and the mixture stirred for 1 h, then the ether was decanted, leaving a tarry residue. More ether was added and the procedure was repeated. The combined extracts were concentrated in vacuo then distilled (55 °C; 0.3 mm Hg) to give 4-acetoxytetrahydrofuran-3-one (13) (2.66 g, 18 mmol; 34%) as a colourless oil. δ_H (80 MHz, $CDCl_3$) 2.10 (3H, s, O_2CCH_3), 3.81 (1H, t, J 8 Hz, H-5), 4.05 (2H, s, H-2), 4.54 (1H, t, J 8 Hz, H-5), 5.20 (1H, t, J 8 Hz, H-4) ppm.

4-Acetoxy-2-bromotetrahydrofuran-3-one (8).

A mixture of 4-acetoxytetrahydrofuran-3-one (13) (3.0 g, 21 mmol) and N-bromosuccinimide (4.08 g, 23 mmol) in dry carbon tetrachloride (100 ml) was stirred for 1h, under an atmosphere of argon, next to a 500 W tungsten lamp. The

mixture was filtered, and the filtrates concentrated *in vacuo* to give 4-acetoxy-2-bromotetrahydrofuran-3-one (8) (4.5 g, 0.20 mmol; 97%) as a colourless oil, which slowly decomposed on standing. δ_H (80 MHz, $CDCl_3$) 2.18 (1.5H, s, O_2CCH_3), 2.22 (1.5H, s, O_2CCH_3), 4.23 (1H, m, H-5), 4.72 (1H, m, H-5), 5.35 (1H, m, H-4), 6.60 (1H, s, H-2) ppm.

Phloroglucinol tribenzenesulphonate (15).

Phloroglucinol dihydrate (16) (100 g, 0.617 mol) and benzenesulphonyl chloride (250 ml, 1.95 mol) were mixed with water (1000 ml) and mechanically stirred. Powdered calcium hydroxide was then added slowly, until the mixture was permanently alkaline to litmus. The mixture was stirred for a further one hour and the crude product obtained by filtration was crystallised from methanol, giving phloroglucinol tribenzenesulphonate (15) (226 g, 0.413 mol; 67%) as tan crystals, m.p. 114-117 °C (lit.[24] m.p. 122 °C).

Phloroglucinol monobenzenesulphonate (17).

To a solution of phloroglucinol tribenzenesulphonate (15) (32.0 g, 59 mmol) in methanol (100 ml) was added methanolic potassium hydroxide solution (19.6 g, 0.349 mol, dissolved in 100 ml of 90% methanol-10% water) with stirring, over a period of 15 min. The mixture was then heated at reflux for 30 min., then cooled and water (1000 ml) added. Concentrated hydrochloric acid was added dropwise to pH 1, and the solution was decolourised by the addition of a few crystals of sodium thiosulphate. The product crystallised from solution overnight, and the solid

was removed by filtration, washed with water, air dried, then azeotroped with benzene, giving phloroglucinol monobenzenesulphonate (17) (9.05 g, 34 mmol; 58%) as off-white crystals, m.p. 141-151 °C (lit.[5] m.p. 158-165 °C).

Phloroglucinol dibenzyl ether monobenzenesulphonate (18).

To a solution of phloroglucinol monobenzenesulphonate (17) (48.0 g, 0.180 mol) in dry DMF (740 ml) was added powdered, oven-dried potassium iodide (88.0 g, 0.530 mol), anhydrous potassium carbonate (178 g, 1.29 mol) and benzyl chloride (69.9 g, 0.552 mol). The mixture was stirred overnight under an atmosphere of nitrogen, then diluted with methylene chloride (1400 ml) and filtered through Celite. The filtrates were concentrated <u>in vacuo</u> to give a red-brown oil, which was dissolved in ethyl acetate and washed with water, then brine, and dried over anhydrous magnesium sulphate. Filtration and concentration of the filtrates <u>in vacuo</u> afforded phloroglucinol dibenzyl ether monobenzenesulphonate (18) (79.0 g, 0.176 mol; 98%) which gave pale crystals from methanol, m.p. 52-56 °C (lit.[5] m.p. 58-62 °C); δ_H (80 MHz, $CDCl_3$) 4.79 (4H, s, 2x Ar-C<u>H</u>$_2$-O), 6.13 (2H, d, J 2 Hz, 2x Ar-<u>H</u>), 6.35 (1H, t, J 2 Hz, Ar-<u>H</u>), 7.22 (10H, s, 10x Ar-<u>H</u>), 7.43 (5H, m, 5x Ar-<u>H</u>) ppm.

Phloroglucinol dibenzyl ether (9).

Methanolic potassium hydroxide solution (296 g, 5.27 mol, dissolved in 1100 ml of 90% methanol-10% water) was added to a suspension of phloroglucinol dibenzyl ether monobenzenesulphonate (18) (79.0 g, 0.177 mol) in methanol

(300 ml). The mixture was heated at reflux for 6 h, then concentrated in vacuo, giving phloroglucinol dibenzyl ether (9) (44.5 g, 0.145 mol; 82%) which gave white needles from carbon tetrachloride, m.p. 85-88 °C (lit.[5] m.p. 90-92 °C); δ_H (80 MHz, $CDCl_3$) 4.95 (4H, s, 2x Ar-C<u>H</u>$_2$-O), 6.08 (2H, d, J 2 Hz, 2x Ar-<u>H</u>), 6.21 (1H, t, J 2 Hz, Ar-<u>H</u>), 7.27 (10H, s, 10x Ar-<u>H</u>) ppm.

3-Acetoxy-3a-bromo-4,6-bis(benzyloxy)-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran (7).

A solution of phloroglucinol dibenzyl ether (9) (0.50 g, 1.63 mmol) in dry methylene chloride (2 ml) was added to dry methylene chloride (10 ml) through which a stream of HBr gas had been passed for 5 min. 4-Acetoxy-2-bromotetrahydrofuran (8) (0.56 g, 2.51 mmol) in dry methylene chloride (2 ml) was then added dropwise over 5 min., under an atmosphere of argon. The solution was stirred at room temperature for a further 20 min., then filtered through a slurry of silica (70-230 mesh) in methylene chloride, and washed through with more methylene chloride. The combined filtrates were then washed with saturated sodium bicarbonate solution, then saturated brine, and dried ($MgSO_4$). Concentration of the solution in vacuo gave a dark oil, which was purified by preparative tlc (83% ethyl acetate - 17% (40-60) petroleum ether) and the band at R_f 0.4 gave the tricyclic bromide (7) (0.20 g, 0.4 mmol; 24%) as a crystalline solid, m.p. 139-142 °C (lit.[5] m.p. 146-147 °C); δ_H (80 MHz, $CDCl_3$) 2.16 (3H, s, $O_2CC\underline{H}_3$), 3.98 (2H, m, <u>H</u>-2), 4.98 (2H, s, Ar-C<u>H</u>$_2$-O), 5.15 (2H, s, Ar-C<u>H</u>$_2$-

O), 5.89 (1H, br, H-3), 6.17 (2H, s, 2x Ar-H), 6.28 (1H, s, H-8a), 7.40 (10H, s, 10x Ar-H) ppm.

The reaction was repeated on larger scale (9.2 times) and the crude product purified by column chromatography on silica gel (60-120 mesh) using 83% ethyl acetate - 17% (40-60) petroleum ether as eluent. This gave the bromide (7) (6%), followed by 3-acetoxy-4,6-bis(benzyloxy)-3a-hydroxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran (19) (0.20 g, 0.45 mmol; 2%), which gave white needles from ethanol, m.p. 146.5-149 °C, (Found: C, 69.51; H, 5.40. $C_{26}H_{24}O_7$ requires C, 69.63; H, 5.41%); ν max ($CHCl_3$) 3570, 1740, 1625, 1605, 1495 cm^{-1}; λ max (MeOH) 217, 238, 270 nm (ϵ 41800, 10300, 1300); δ_H 2.17 (3H, s, O_2CCH_3), 2.89 (1H, br, OH), 4.02 (1H, A of ABX, J_{AB} 10 Hz, J_{AX} 2 Hz, H-2), 4.07 (1H, B of ABX, J_{BA} 10 Hz, H-2), 4.98 (2H, s, Ar-CH_2-O), 5.12 (2H, s, Ar-CH_2-O), 5.58 (1H, d, J_{XA} 2 Hz, H-3), 5.93 (1H, s, H-8a), 6.17 (2H, AB, J_{AB} 2Hz, 2x Ar-H), 7.38 (10H, s, 10x Ar-H) ppm; m/z 448 (M^+), 333, 271.

The spent column was washed with acetone, and the washings concentrated in vacuo to a dark solid, which was recrystallised from methanol to give 3,3a-acetonide-4,6-bis(benzyloxy)-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran (20) (84 mg, 0.19 mmol; 1%), δ_H (80 MHz, $CDCl_3$) 1.25 (3H, s, CH_3), 1.48 (3H, s, CH_3), 4.00 (1H, A of ABX, J_{AB} 10.8 Hz, J_{AX} 2.3 Hz, H-2), 4.22 (1H, B of ABX, J_{BA} 10.8 Hz, H-2), 4.75 (1H, d, J_{XA} 2.3 Hz, H-3), 4.99 (2H, s, Ar-CH_2-O), 5.04 (2H, s, Ar-CH_2-O), 5.96 (1H, s, H-8a), 6.12 (1H, A of AB, J_{AB} 2.0 Hz, Ar-H), 6.21 (1H, B of AB, J_{AB} 2.0 Hz, Ar-

H), 7.37 (10H, s, 10x Ar-H) ppm; m/z 446 (M^+), 181, 180, 91.

Condensation of phenol (9) and bromide (8) using hydrogen chloride.

A solution of phloroglucinol dibenzyl ether (9) (117 mg, 0.38 mmol) in dry methylene chloride (2 ml) was added to dry methylene chloride (6 ml) through which a stream of HCl gas had been passed for 5 min. 4-Acetoxy-2-bromotetrahydrofuran (8) (166 mg, 0.71 mmol) in dry methylene chloride (3 ml) was then added dropwise over 5 min., under an atmosphere of argon. The solution was stirred at room temperature for a further 30 min., then filtered through a slurry of silica (70-230 mesh) in methylene chloride, and washed through with more methylene chloride. The combined filtrates were then washed with saturated sodium bicarbonate solution, then saturated brine, and dried ($MgSO_4$). Concentration of the solution in vacuo gave a dark oil, which was purified by preparative tlc (83% ethyl acetate - 17% (40-60) petroleum ether). The product (30 mg) gave m/z 512, 510, 431.

Condensation of phenol (9) and bromide (8) using trifluoroacetic acid.

Bromide (8) (152 mg, 0.68 mmol), dissolved in dry methylene chloride (7 ml), was added dropwise, under an atmosphere of argon, to a solution of phloroglucinol dibenzyl ether (9) (212 mg, 0.69 mmol) and trifluoroacetic acid (1 drop) in dry methylene chloride (5 ml). The solution was stirred at room temperature for 22 h., then

concentrated in vacuo to a dark solid, which was purified by preparative tlc (83% ethyl acetate - 17% (40-60) petroleum ether) to give the tricyclic bromide (7) (56 mg, 0.11 mmol; 16%).

3a-Bromo-4,6-bis(benzyloxy)-3-hydroxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran (23).

A suspension of tricyclic bromide (7) (50 mg, 0.10 mmol) in dry ether (10 ml) was slowly added to a stirred suspension of lithium aluminium hydride (25 mg, 0.66 mmol) in dry ether (10 ml). The mixture was stirred at reflux for 1h., then quenched with wet ether (10 ml), followed by 2M sulphuric acid (15 ml). The ether layer was separated, washed with water, then dried ($MgSO_4$) and concentrated in vacuo. The crude product was purified by preparative tlc (2% methanol - 98% chloroform) to give 3a-bromo-4,6-bis(benzyloxy)-3-hydroxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran (23) (18 mg, 0.04 mmol; 39%), ν max (nujol) 3300, 1630 cm^{-1}; δ_H (80 MHz, $CDCl_3$) 2.58 (1H, br, OH), 3.98 (2H, m, H-2), 4.62 (1H, d, J 2 Hz, H-3), 4.98 (2H, s, Ar-CH_2-O), 5.10 (2H, s, Ar-CH_2-O), 6.17 (2H, s, 2x Ar-H), 6.29 (1H, s, 8a-H), 7.37 (5H, s, 5x Ar-H), 7.39 (5H, s, 5x Ar-H) ppm.

3-Acetoxy-4,6-bis(benzyloxy)-2,3,3a,8a-tetrahydrofuro-[2,3-b]benzofuran (24).

A solution of tributyltin hydride (40 mg, 0.14 mmol) in dry benzene (4 ml) was added to a stirred solution of tricyclic bromide (7) (48 mg, 0.09 mmol), under an atmosphere of argon. Azobisdiisobutyronitrile (5 mg) was

added as an initiator, and the solution was heated at reflux for 100 min. The solvent was then evaporated *in vacuo*, and the residue was applied to preparative tlc plates and eluted in 50% benzene - 50% methylene chloride. The band with R_f value of 0.26 was removed, to give *3-acetoxy-4,6-bis(benzyloxy)-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran* (24) (20 mg, 0.05 mmol; 49%) which was recrystallised from ethanol to form colourless crystals, m.p. 132-134 °C (Found: C, 72.70; H, 5.68. $C_{26}H_{24}O_6$ requires C, 72.21; H, 5.59%); ν max ($CHCl_3$) 1735, 1625, 1605 cm^{-1}; δ_H (200 MHz; $CDCl_3$) 2.08 (3H, s, O_2CCH_3), 3.86 (1H, A of ABX, J_{AB} 10.9 Hz, J_{AX} 2.8 Hz, H-2), 4.01 (1H, d, J 5 Hz, H-3a), 4.05 (1H, B of ABX, J_{BA} 10.7 Hz, H-2), 4.97 (2H, s, Ar-CH_2-O), 5.07 (2H, AB, Ar-CH_2-O), 5.48 (1H, d, J_{XA} 2.6 Hz, H-3), 6.14 (1H, d, J 2.0 Hz, Ar-H), 6.16 (1H, d, J 2.0 Hz, Ar-H), 6.41 (1H, d, J 5.7 Hz, H-8a), 7.37 (10H, m, 10x Ar-H) ppm; m/z 432 (M^+), 293, 91.

3-Acetoxy-4-benzyloxy-6-hydroxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran (25).

The acetate (24) (46 mg, 0.12 mmol) was dissolved in ethyl acetate (15 ml). 5% palladium on carbon (66 mg) was added, and the mixture was stirred under an atmosphere of hydrogen for 5 h. The mixture was then filtered through Celite, and the filtrates concentrated *in vacuo*. The crude product mixture was purified by preparative tlc (5% acetone - 95% methylene chloride), and the band with R_f value of 0.42 gave *3-acetoxy-4-benzyloxy-6-hydroxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran* (25) (16 mg, 0.05 mmol;

44%) which gave white crystals from ethanol, m.p. 177-179 °C (M^+: 342.11033. $C_{19}H_{18}O_6$ requires 342.11034.); ν max ($CHCl_3$) 3250, 1735, 1620 cm^{-1}; δ_H (80 MHz, $CDCl_3$) 2.08 (3H, s, $O_2CC\underline{H}_3$), 3.91 (1H, A of ABX, J_{AB} 11 Hz, J_{AX} 2.6 Hz, H-2), 4.00 (1H, d, J 5.1 Hz, H-3a), 4.05 (1H, B of ABX, J_{BA} 11 Hz, H-2), 5.07 (2H, s, Ar-$C\underline{H}_2$-O), 5.45 (1H, d, J_{XA} 2.4 Hz, H-3), 5.99 (2H, AB, J_{AB} 1.9 Hz, 2x Ar-H), 6.40 (1H, d, J 5.7 Hz, H-8a), 7.36 (5H, s, 5x Ar-H) ppm; m/z 342 (M^+), 282, 206, 191, 91.

This reaction was repeated using a hydrogenation time of 20 h. Preparative tlc of the product mixture (5% acetone - 95% methylene chloride) gave three products.

The band with R_f value 0.20 gave 3-acetoxy-4,6-dihydroxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran (27%), which recrystallised form chloroform to give white crystals, m.p. 162-164 °C (M^+: 252.06338. $C_{12}H_{12}O_6$ requires 252.06339.); δ_H (80 MHz, d_6-acetone) 2.07 (3H, s, $O_2CC\underline{H}_3$), 3.86 (1H, A of ABX, J_{AB} 11 Hz, J_{AX} 3 Hz, H-2), 3.91 (1H, d, J 6 Hz, H-3a), 3.99 (1H, B of ABX, J_{BA} 11 Hz, H-2), 5.12 (1H, d, J_{XA} 3 Hz, H-3), 5.80 (1H, d, J 2 Hz, Ar-H), 5.91 (1H, d, J 2 Hz, Ar-H), 6.33 (1H, d, J 6 Hz, H-8a), 8.19 (1H, br, O$\underline{H}$) ppm; m/z 252 (M^+), 192, 163.

The band of R_f value 0.42 gave phenol (25) (24%), and the band with R_f of 0.64 gave 3-acetoxy-6-benzyloxy-4-hydroxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran (26) (10%) which was recrystallised from ethanol, forming off-white crystals, m.p. 147-151 °C (M^+: 342.11033. $C_{19}H_{18}O_6$ requires 342.11034.); δ_H (80 MHz, $CDCl_3$) 2.19 (3H, s,

O_2CCH_3), 3.80 (1H, d, J 6 Hz, H-3a), 4.06 (1H, A of ABX, J_{AB} 12 Hz, J_{AX} 4 Hz, H-2), 4.20 (1H, B of ABX, J_{BA} 12 Hz, H-2), 4.86 (1H, d, J_{XA} 4 Hz, H-3), 4.98 (2H, s, Ar-CH_2-O), 6.05 (1H, d, J 2 Hz, Ar-H), 6.15 (1H, d, J 2Hz, Ar-H), 6.42 (1H, d, J 6 Hz, H-8a), 7.36 (5H, s, 5x Ar-H) ppm; m/z 342 (M^+), 282, 206, 191, 91.

3-Acetoxy-4-benzyloxy-6-methoxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran (27).

To a solution of phenol (25) (25 mg, 0.07 mmol) in dry acetone (5 ml), was added anhydrous potassium carbonate (16 mg) and methyl iodide (0.02 ml, 0.35 mmol). The slurry was stirred under an atmosphere of argon for 4 days, then the mixture was filtered, and the filtrates concentrated in vacuo, affording 3-acetoxy-4-benzyloxy-6-methoxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran (27) (26 mg, 0.07 mmol; 100%) which recrystallised from ethyl acetate - (40-60) petroleum ether as white crystals, m.p. 158-160 °C (M^+: 356.1260. $C_{20}H_{20}O_6$ requires 356.12599.); δ_H 2.08 (3H, s, O_2CCH_3), 3.72 (3H, s, OCH_3), 3.89 (1H, A of ABX, J_{AB} 11 Hz, J_{AX} 2.6 Hz, H-2), 3.99 (1H, B of ABX, J_{BA} 11 Hz, H-2), 4.01 (1H, d, J 6 Hz, H-3a), 5.08 (2H, s, Ar-CH_2-O), 5.48 (1H, d, J_{XA} 2.5 Hz, H-3), 6.06 (2H, s, 2x Ar-H), 6.41 (1H, d, J 6 Hz, H-8a), 7.37 (5H, s, 5x Ar-H) ppm; m/z 356 (M^+), 296, 205, 91.

5,7-Dihydroxy-4-methylcoumarin (30).

A suspension of phloroglucinol dihydrate (200 g, 1.23 mol) in ethyl acetoacetate (158 g, 1.22 mol) was added via pipette, over 30 min., to stirred, ice-cooled, concentrated

sulphuric acid (750 ml). The suspension was allowed to stand at room temperature for 2 days, then was poured onto ice-water (3000 ml) and filtered. The solid was recrystallised from acetic acid, giving 5,7-dihydroxy-4-methylcoumarin monohydrate (30) (224 g, 86%), m.p. >280 °C (lit.[10] m.p. 296 °C).

5-Hydroxy-7-methoxy-4-methylcoumarin (31).

5,7-Dihydroxy-4-methylcoumarin monohydrate (30) (100 g, 0.48 mol) was added to a solution of sodium carbonate (100 g) in water (900 ml) and the mixture was heated with stirring to 80 °C, by which temperature the solid had dissolved. Dimethyl sulphate (75 ml, 0.79 mol) was then added dropwise over 40 min., whilst maintaining the temperature at 80 °C. On cooling to 50 °C, the mixture was filtered. The solid was suspended in 5% sodium hydroxide (700 ml) and refiltered. This was repeated, and the solid was then recrystallised from methanol to give 5,7-dimethoxy-4-methylcoumarin (5.01 g, 0.02 mol; 5%) as fine white needles, m.p. 167-169 °C (lit.[9] m.p. 169-171 °C).

The combined alkaline filtrates were acidified (conc. HCl) and filtered to give a pale foam, which was sucked as dry as possible. This solid was recrystallised from ethanol, affording 5-hydroxy-7-methoxy-4-methylcoumarin (31) (22.5 g, 0.11 mol; 23%) as off-white crystals, m.p. 251-254 °C (lit.[9] m.p. 255-256 °C).

5-Benzyloxy-7-methoxy-4-methylcoumarin (32).

A solution of 5-hydroxy-7-methoxy-4-methylcoumarin (31) (20 g, 97 mmol) and benzyl chloride (18.8 g, 148 mmol)

in dry acetone (1500 ml), containing sodium iodide (19 g) and anhydrous sodium carbonate (30 g), was stirred at reflux for 7 h., then at room temperature for a further 17 h. The salts were removed by filtration, and the filtrates were concentrated in vacuo to a red oil, which was triturated from ethanol to give 5-benzyloxy-7-methoxy-4-methylcoumarin (32) (21.7 g, 74 mmol; 76%) as white crystals, m.p. 139-143 °C (lit.[9] m.p. 141-142 °C); δ_H (80 MHz, $CDCl_3$) 2.46 (3H, d, J 2 Hz, CH_3), 3.82 (3H, s, OCH_3), 5.08 (2H, s, Ar-CH_2-O), 5.92 (1H, q, J 2 Hz, H-3), 6.36 (1H, d, J 2.3 Hz, Ar-H), 6.44 (1H, d, J 2.3 Hz, Ar-H), 7.25 (5H, s, 5x Ar-H) ppm.

5-Benzyloxy-7-methoxy-4-formylcoumarin (28).

A stirred mixture of 5-benzyloxy-7-methoxy-4-methylcoumarin (32) (21.7 g, 73 mmol) and selenium dioxide (20 g, 180 mmol) in xylene (700 ml) was heated under reflux, using a Dean-Stark trap, for 10 h. The mixture was allowed to cool slightly, then filtered, and the filtrates concentrated in vacuo to a brown solid. The solid was dissolved in hot methylene chloride, then filtered to remove residual selenium. The filtrates were then concentrated in vacuo, giving 5-benzyloxy-7-methoxy-4-formylcoumarin (28) (22.0 g, 71 mmol; 97%), which gave yellow needles from benzene, m.p. 184-187 °C (lit.[9] m.p. 189-191 °C); δ_H (200 MHz, $CDCl_3$) 3.85 (3H, s, OCH_3), 5.15 (2H, s, Ar-CH_2-O), 6.26 (1H, s, H-3), 6.42 (1H, d, J 2.2 Hz, Ar-H), 6.47 (1H, d, J 2.2 Hz, Ar-H), 7.37 (5H, s, 5x Ar-H), 10.40 (1H, s, CHO) ppm.

4-Benzyloxy-6-methoxy-2-oxo-tetrahydrofuro[2,3-b]benzofuran (29).

A mixture of 5-benzyloxy-7-methoxy-4-formylcoumarin (28) (22.0 g, 71 mmol) and glacial acetic acid (300 ml) was heated, with stirring, to 100 °C, by which time the solid had dissolved. Zinc dust (20.0 g, 306 mmol) was then added cautiously, and the mixture was stirred at 120 °C for 2 h. After cooling to ca. 60 °C, chloroform (300 ml) was added, and the mixture was filtered. The filtrates were concentrated in vacuo to a pale solid, which was redissolved in chloroform then washed with water, dried ($MgSO_4$) and concentrated in vacuo to give 4-benzyloxy-6-methoxy-2-oxo-tetrahydrofuro[2,3-b]benzofuran (29) (22.3 g, 71 mmol; 100%). A sample was recrystallised from methanol, giving white crystals, m.p. 164-166 °C (lit.[9] m.p. 166-167 °C); δ_H (80 MHz, $CDCl_3$) 2.92 (2H, d, J 6 Hz, H-3), 3.74 (3H, s, CH_3), 4.15 (1H, q, J 6 Hz, H-3a), 5.04 (2H, s, Ar-CH_2-O), 6.15 (2H, s, 2x Ar-H), 6.48 (1H, d, J 6 Hz, H-8a), 7.37 (5H, s, 5x Ar-H) ppm.

2-Hydroxy-4-benzyloxy-6-methoxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran (33).

To a solution of the tricyclic lactone (29) (48 mg, 0.15 mmol) in dry toluene (5 ml) was added a 1.5 M solution of diisobutylaluminium hydride (0.11 ml, 0.165 mmol), and stirred at -20 °C for 1 h. On warming to room temperature, 2 M hydrochloric acid (5 ml) was added, and the mixture was allowed to stir for 30 min. The toluene layer was separated and dried ($MgSO_4$), then concentrated in vacuo. The crude

product was then purified by preparative tlc (5% acetone - 95 % methylene chloride); the band with R_f 0.3 giving 2-hydroxy-4-benzyloxy-6-methoxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran (33) (23 mg, 0.07 mmol; 48%) as a white solid. ν_{max} (nujol) 3390, 1630, 1610 cm^{-1}.

2-Acetoxy-4-benzyloxy-6-methoxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran (34).

Anhydrous sodium acetate (3.4 g) and acetic anhydride (5 ml) were added to a solution of the tricyclic hemiacetal (33) (531 mg, 169 mmol) in benzene (70 ml). The mixture was stirred at room temperature overnight, then at reflux for 1 h. On cooling, the solids were removed by filtration, and the filtrate was washed with water, then dried ($MgSO_4$) and concentrated in vacuo, giving the tricyclic acetate (34) (603 mg, 169 mmol; 100%) as a pale orange oil which slowly crystallised. ν_{max} (film) 1745, 1620, 1500 cm^{-1}.

Flash vacuum pyrolysis of tricyclic acetate (34).

Tricyclic acetate (34) (27 mg, 0.08 mmol) was placed in a sublimation thimble which was then attached to the flash vacuum pyrolysis apparatus. The apparatus was evacuated (0.001 mm Hg), and the thermolysis tube was heated to 500 °C. The sublimation thimble was then heated to 200 °C for 2 h., and the products were collected in a liquid nitrogen trap. The product mixture was subjected to preparative tlc (10% ethyl acetate - 90% (40-60) petroleum ether), and the band with R_f 0.6 gave 4-benzyloxy-6-methoxy-3a,8a-dihydrofuro[2,3-b]benzofuran (35) (11 mg, 0.04 mmol; 50%) which gave white crystals from methanol,

m.p. 97.5-98.5 °C (M^+: 296.1049. $C_{18}H_{16}O_4$ requires 296.10486.); ν_{max} 1625, 1605, 1500 cm^{-1}; δ_H (80 MHz, $CDCl_3$) 3.73 (3H, s, $C\underline{H}_3$), 4.56 (1H, dt, J 7, 3 Hz, $\underline{H}$-3a), 5.05 (2H, s, Ar-$C\underline{H}_2$-O), 5.31 (1H, t, J 3 Hz, $\underline{H}$-3), 6.08 (1H, A of AB, J_{AB} 2 Hz, Ar-$\underline{H}$), 6.14 (1H, B of AB, J_{BA} 2 Hz, Ar-$\underline{H}$), 6.41 (1H, t, J 3 Hz, $\underline{H}$-2), 6.66 (1H, d, J 6 Hz, $\underline{H}$-8a), 7.38 (5H, s, 5x Ar-$\underline{H}$) ppm; m/z 296 (M^+), 267, 205, 177, 91.

Vertical flash vacuum pyrolysis of acetate (34).

Tricyclic acetate (34) (586 mg, 1.65 mmol) was dissolved in chloroform (25 ml), and Celite (4 g) was added. The solvent was removed in vacuo to give a fine powder. The powder was placed in a flask at the top of a vertical flash vacuum pyrolysis system. The apparatus was evacuated (0.01 mm Hg) and the silica tube heated to 500 °C. The flask was then tapped gently to slowly introduce the dust to the top of the pyrolysis column, and the products were collected from the liquid nitrogen trap when all the dust had been used. The crude product was purified by dry flash chromatography, using a gradient of 0 - 30% ethyl acetate in (40-60) petroleum ether, giving the acetate elimination product (35) (71 mg, 0.24 mmol; 14%).

Pyrolysis of acetate (34).

A solution of tricyclic acetate (34) (228 mg, 0.64 mmol) in toluene (20 ml) was dropped slowly onto a heated (400 °C) column of glass beads, under a slow flow of nitrogen, and the products were collected in a liquid nitrogen trap. After the reaction was complete, the column was allowed to cool, and the glass beads were washed with

acetone. The acetone washings were combined with the toluene solution from the trap, and concentrated *in vacuo* to a dark solid, which was purified by preparative tlc (20% ethyl acetate - 80% (40-60) petroleum ether), giving the elimination product (35) (58 mg, 0.20 mmol; 30%).

4-Hydroxy-6-methoxy-3a,8a-dihydrofuro[2,3-b]benzofuran (5).

Benzyl ether (35) (66 mg, 22mmol) was dissolved in a mixture of ether (10 ml) and liquid ammonia (40 ml). Sodium metal (50 mg, 2.2 mmol) was added, with stirring, in four portions, over a period of 10 min., in order to maintain a blue solution. Stirring was continued for a further 5 min., then excess sodium was destroyed by the addition of ammonium chloride. The ammonia and ether were allowed to evaporate in the fume hood, then the residue was partitioned between ethyl acetate and water. The organic layer was dried ($MgSO_4$), and concentrated *in vacuo*, affording 4-hydroxy-6-methoxy-3a,8a-dihydrofuro[2,3-b]benzofuran (5) (38 mg, 0.18 mmol; 83%) as an off-white solid. The product was further purified by preparative tlc (20% ethyl acetate - 80% (40-60) petroleum ether), giving a white solid, m.p. 128.5-130 °C (lit.[4] m.p. 128-130 °C), (M^+: 206.0579. $C_{11}H_{10}O_4$ requires 206.05791); ν max ($CHCl_3$) 3590, 3285, 1630, 1510 cm^{-1}; δ_H (80 MHz, $CDCl_3$) 3.72 (3H, s, OCH_3), 4.54 (1H, dt, J 7, 2 Hz, H-3a), 4.89 (1H, br, OH), 5.32 (1H, t, J 2 Hz, H-3), 5.91 (1H, d, J 2 Hz, Ar-H), 6.11 (1H, d, J 2 Hz, Ar-H), 6.43 (1H, t, J 2 Hz, H-2), 6.67 (1H, d, J 7 Hz, H-8a) ppm; m/z 206 (M^+), 178, 163, 149,

134.

2,4-Diacetoxy-6-methoxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran (37).

Tricyclic acetate (34) (140 mg, 0.39 mmol) was dissolved in ethyl acetate, and 10% palladium on carbon (53 mg) was added. The mixture was stirred under an atmosphere of hydrogen for 2.5 h., then filtered through Celite. The filtrates were concentrated in vacuo, and the residue was dissolved in benzene (20 ml) and acetic anhydride (1.5 ml). Anhydrous sodium acetate (0.8 g) was added, and the mixture was stirred at room temperature for 19 h. The mixture was then filtered to remove salts, and the filtrates were concentrated in vacuo, giving 2,4-diacetoxy-6-methoxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran (37) (86 mg, 0.28 mmol; 71%) as an oil. The diastereomeric mixture was not resolved, but used immediately in pyrolysis reactions.

Pyrolysis of diacetate (37).

A solution of diacetate (37) (86 mg, 0.28 mmol) in toluene (20 ml) was dropped slowly onto a heated (400 °C) column of glass beads, under a slow flow of nitrogen. On cooling, the column was washed with acetone, and the washings added to the toluene solution, obtained from the liquid nitrogen trap. The solution was concentrated in vacuo, and the residue was purified by preparative tlc (20% ethyl acetate - 80% (40-60) petroleum ether), to give 4-acetoxy-6-methoxy-3a,8a-dihydrofuro[2,3-b]benzofuran (38) (23 mg, 0.09 mmol; 33%), δ_H (80 MHz, $CDCl_3$) 2.30 (3H, s, $O_2CC\underline{H}_3$), 3.72 (3H, s, $OC\underline{H}_3$), 4.45 (1H, dt, J 7, 2.5 Hz, H-

3a), 5.15 (1H, t, J 2.5 Hz, H-3), 6.20 (1H, A of AB, J_{AB} 2 Hz, Ar-H), 6.38 (1H, B of AB, J_{BA} 2 Hz, Ar-H), 6.42 (1H, t, J 2.5 Hz, H-2), 6.68 (1H, d, J 7 Hz, H-8a) ppm.

4-Hydroxy-6-methoxy-3a,8a-dihydrofuro[2,3-b]benzofuran (5).

An aqueous solution of potassium carbonate (100mg in 1 ml) was added to a solution of acetate (38) (22 mg, 0.09 mmol) in methanol (1.5 ml). The solution was allowed to stand at room temperature for 1 h., then acidified (10% HCl), and extracted with ethyl acetate. The organic layer was washed with water, then dried ($MgSO_4$), and concentrated in vacuo to give MW206 (5) (18 mg, 0.09 mmol; 100%).

4-Hydroxy-6-methoxy-2,3,3a,8a-tetrahydrofuro[2,3-b]-benzofuran (39).

To a solution of 4-benzyloxy-6-methoxy-3a,8a-dihydrofuro[2,3-b]benzofuran (35) (58 mg, 0.20 mmol) in ethyl acetate (15 ml), was added 10% palladium on carbon (100 mg), and the mixture was stirred under an atmosphere of hydrogen for 4 h. The mixture was then filtered through Celite, and the filtrates concentrated in vacuo. The product was purified by preparative tlc (5% acetone - 95% methylene chloride), affording 4-hydroxy-6-methoxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran (39) (34 mg, 0.16 mmol; 83%) as a white solid, m.p. 150-152 °C (lit.[11] m.p. 152 °C); δ_H (80 MHz, $CDCl_3$) 2.16 (2H, m, H-3), 3.64 (1H, m, H-3a), 3.71 (3H, s, OCH_3), 4.04 (2H, m, H-2), 5.11 (1H, br, OH), 5.91 (1H, A of AB, J_{AB} 2 Hz, Ar-H), 6.02 (1H, B of AB, J_{BA} 2 Hz, Ar-H), 6.30 (1H, d, J 6 Hz, H-8a) ppm.

3-Bromocyclopentenone (41).

A 1 M solution of bromine in benzene (2.2 ml, 2.2 mmol) was added to an ice-cold, stirred solution of triphenylphosphine (574 mg, 2.2 mmol) in benzene (20 ml), giving a white suspension of triphenylphosphonium bromide. Triethylamine (221 mg, 2.2 mmol) and 1,3-cyclopentanedione (194 mg, 2.0 mmol) was then added, and the mixture was stirred at room temperature for 4 h., then filtered through a short column of silica (230-400 mesh; 5 g), which was then washed with ether. The combined filtrates were concentrated <u>in vacuo</u> to give 3-bromocyclopentenone (41) (257 mg, 1.6 mmol; 80%) as a colourless oil. δ_H (60 MHz, $CDCl_3$) 2.60 (2H, m, <u>H</u>-5), 3.00 (2H, m, <u>H</u>-4), 6.40 (1H, t, J 1.5 Hz, <u>H</u>-2) ppm.

Methyl succinyl chloride (42).

Methyl hydrogen succinate (43) (132 g, 1.0 mol) was dissolved in thionyl chloride (145 ml) and the solution was warmed to 40 °C for 3h. Excess thionyl chloride was then removed at the water pump, and the residue was distilled (80 °C, 23 mmHg), to give methyl succinyl chloride (42) (102 g, 0.67 mol; 67%).

Methyl <u>t</u>-butyl succinate (44).

Methyl succinyl chloride (42) (101 g, 0.67 mol) was dissolved in chloroform (200 ml) and added dropwise to a stirred solution of <u>t</u>-butanol (120 g, 1.62 mol) in N,N-dimethylaniline (117 g, 0.96 mol). After addition was complete, the black mixture was heated at reflux for 2.5 h. The mixture was poured onto ice, then adjusted to pH 1

using 2 M sulphuric acid. The chloroform layer was reserved, then the aqueous layer was extracted with methylene chloride (3 x 200 ml), and the combined organic layers were washed with 2 M sulphuric acid (5 x 150 ml), saturated sodium bicarbonate solution (250 ml), then water (250 ml). The organic layer was then dried ($MgSO_4$), and concentrated in vacuo to a black oil. Distillation (52 °C, 0.5 mmHg) gave methyl t-butyl succinate (44) (88.4 g, 0.47 mol; 70%) as a clear liquid. ν max (film) 2985, 1728, 1438, 1365, 1145 cm^{-1}.

t-Butyl hydrogen succinate (45).

Methyl t-butyl succinate (44) (80 g, 0.42 mol) was added to a solution of sodium hydroxide (17.0 g, 0.42 mol) in water (950 ml) and 1,4-dioxan (900 ml), and stirred at room temperature for 40 h. The dioxan was removed in vacuo, and the resulting aqueous solution was washed with ether. The aqueous layer was added to ice, and acidified with 2 M sulphuric acid, then extracted with ether (6 x 250 ml). The combined ethereal extracts were washed with water, then dried ($MgSO_4$), and concentrated to an oil. Distillation (90 °C, 0.5 mmHg) of the oil gave t-butyl hydrogen succinate (45) (32.5 g, 0.19 mol; 44%) as a colourless oil, which crystallised. δ_H (60 MHz, d_6-acetone) 1.45 (9H, s, 3x CH_3), 2.55 (4H, s, 2x $-CH_2-$), 9.45 (1H, br, CO_2H) ppm.

Diethyl 3-carbo-t-butoxypropionylmalonate (47).

Diethylethoxymagnesiomalonate was prepared by cautious addition of a solution of diethyl malonate (24 g, 0.15 mol) in dry ethanol (11 ml) to a stirred mixture of magnesium

turnings (3.7 g, 0.15 mol), carbon tetrachloride (0.5 ml), and dry ethanol (5 ml). After the initial reaction, dry ether (60 ml) was added, and the mixture was heated at reflux for 2 h. The resulting solution was concentrated in vacuo, giving diethylethoxymagnesiomalonate as a viscous oil, which was dissolved in dry ether (60 ml) for later use. Concurrently, the mixed anhydride was made by addition of ethyl chloroformate (16.3 g, 0.15 mol) to a stirred, ice-cooled solution of t-butyl hydrogen succinate (45) (26.5 g, 0.15 mol) and triethylamine (15.2 g, 0.15 mol) in dry toluene (150 ml). The solution was stirred at 0 °C for a further 30 min., then the solution of diethylethoxymagnesiomalonate in ether prepared earlier, was added dropwise. The mixture was allowed to stir at room temperature overnight, then it was poured onto ice, acidified with 2 M sulphuric acid, and extracted with ether. The organic layer was washed with 2 M sulphuric acid, saturated sodium bicarbonate solution, then water, and dried ($MgSO_4$), then concentrated in vacuo to an oil. Distillation of the oil removed residual toluene, diethyl malonate and t-butyl hydrogen succinate, leaving diethyl 3-carbo-t-butoxypropionylmalonate (47) (23.1 g, 0.07 mol; 48%) as a pale yellow oil which was not further purified. ν_{max} (film) 2980, 1730, 1648, 1605, 1368 cm^{-1}.

2-Carbethoxy-4-carbo-t-butoxycyclopentane-1,3-dione (48).

A solution of diethyl 3-carbo-t-butoxypropionylmalonate (47) (15.8 g, 50 mmol) in dry

benzene (50 ml) was added dropwise to a stirred, refluxing suspension of potassium t-butoxide (16.8 g, 150 mmol) in dry benzene (200 ml). The thick slurry was heated at reflux for a further 2 h., then stirred at room temperature overnight. The mixture was poured onto ice, acidified with 2 M sulphuric acid, then extracted into ethyl acetate. The aqueous layer was then saturated with sodium chloride then further extracted with ethyl acetate. The combined organic layers were back-extracted into saturated sodium bicarbonate solution which was then acidified by cautious addition of 2 M sulphuric acid, and subsequently saturated with sodium chloride. This aqueous solution was extracted with ethyl acetate, and the organic layer was washed with brine, then dried ($MgSO_4$), and concentrated in vacuo to give 2-carbethoxy-4-carbo-t-butoxycyclopentane-1,3-dione (48) (6.01 g, 22mmol; 44%) as an orange oil which slowly crystallised. δ_H (60 MHz, $CDCl_3$) 1.43 (3H, t, J 7 Hz, $OCH_2\underline{CH_3}$), 1.53 (9H, s, 3x $C\underline{H_3}$), 3.04 (2H, m, H-5), 3.60 (1H, m, H-4), 4.53 (2H, q, J 7 Hz, $OC\underline{H_2}CH_3$), 11.10 (1H, br, OH) ppm.

2-Carbethoxycyclopentane-1,3-dione (49).

A suspension of 2-carbethoxy-4-carbo-t-butoxycyclopentane-1,3-dione (48) (2.70 g, 10 mmol) and p-toluene sulphonic acid (0.4 g) in benzene (200 ml) dissolved on heating, and the solution was refluxed for 10.5 h. On cooling, the mixture was filtered, and the filtrates were concentrated in vacuo to give crude 2-carbethoxycyclopentane-1,3-dione (49) (1.65 g, 10 mmol;

97%) as a brown solid. Attempted recrystallisations from methanol, methanol-water, and chloroform-(40-60) petroleum ether were unsuccessful. δ_H (60 MHz, $CDCl_3$) 1.29 (3H, t, J 7 Hz, $OCH_2\underline{CH_3}$), 2.65 (4H, s, 2x $-\underline{CH_2}-$), 4.43 (2H, q, J 7 Hz, $O\underline{CH_2}CH_3$) ppm.

3-Bromo-2-carbethoxycyclopentenone (40).

Oxalyl bromide (0.5 ml) was added to an ice-cooled solution of 2-carbethoxycyclopentane-1,3-dione (49) (155 mg, 0.91 mmol) in dry benzene (30 ml) and dry methylene chloride (6 ml), and the solution was stirred at 0 °C for 3 h., protected from atmospheric moisture by a calcium chloride guard tube. The solution was concentrated <u>in vacuo</u> at 0 °C to an oil, which was dissolved in 10% ether - 90% methylene chloride (1 ml), and filtered through a short column of Florosil (100-200 mesh; 3 g). Concentration of the filtrates <u>in vacuo</u> gave 3-bromo-2-carbethoxycyclopentenone (40) (126 mg, 0.54 mmol; 54%) as a pale yellow oil. δ_H (60 MHz, $CDCl_3$) 1.40 (3H, t, J 7 Hz, $OCH_2\underline{CH_3}$), 2.90 (4H, m, 2x $-\underline{CH_2}-$), 4.40 (2H, q, J 7 Hz, $O\underline{CH_2}CH_3$) ppm.

5,7-Dimethoxycyclopentenon[2,3-c]coumarin (51).

To a solution of 3,5-dimethoxyphenol (50) (43 mg, 0.28 mmol) and 2-carbethoxy-3-bromocyclopentenone (40) (30 mg, 0.13 mmol) in methylene chloride (20 ml) was added zinc carbonate (0.5 g) and sodium bicarbonate (1.5 g). The mixture was stirred at reflux for 3 h., under an atmosphere of nitrogen, then overnight at room temperature. The reaction mixture was then filtered and washed with ethyl

acetate. The combined filtrates were evaporated in vacuo, and the crude product was purified by preparative tlc (5% acetone - 95% methylene chloride), the blue fluorescent band giving the coumarin (51) (11 mg, 0.04 mmol; 33%), δ_H (80 MHz, $CDCl_3$) 2.61 (2H, m, H-9), 3.37 (2H, m, H-8), 3.88 (3H, s, OCH_3), 3.92 (3H, s, OCH_3), 6.30 (1H, d, J 2.3 Hz, Ar-H), 6.44 (1H, d, J 2.3 Hz, Ar-H) ppm.

Aflatoxin B_2 (52).

The phenol (39) (42 mg, 0.20 mmol) and bromide (40) (126 mg, 0.54 mmol) were dissolved in methylene chloride (20 ml), and zinc carbonate (0.6 g) and sodium bicarbonate (0.4 g) were added. The mixture was heated, with stirring, at reflux for a total of 12 h., under an atmosphere of nitrogen, then at room temperature overnight. The reaction mixture was then poured into a Soxhlet apparatus, and extracted with ethyl acetate for 3h. The solvent was evaporated in vacuo, and the product was separated by preparative tlc (10% acetone - 90% methylene chloride), and further purified by preparative tlc (70% ethyl acetate - 28% benzene - 2% formic acid), the blue fluorescent band giving racemic aflatoxin B_2 (52) (3 mg, 0.01 mmol; 5%), δ_H (200 MHz, $CDCl_3$) 2.28 (2H, m, H-9), 2.63 (2H, m, H-2), 3.39 (2H, m, H-3), 3.63 (1H, m, H-9a), 3.94 (3H, s, OCH_3), 4.16 (2H, m, H-8), 6.33 (1H, s, Ar-H), 6.47 (1H, d, J 5.6 Hz, H-6a) ppm.

2-Carbethoxy-3-(2,4,6-trimethoxyphenyl)cyclopent-2-enone (54).

Phosphorus pentoxide (0.5 g) was added to a solution

of 1,3,5-trimethoxybenzene (30 mg, 0.18 mmol) and dione (49) (30 mg, 0.18 mmol) in a mixture of dry benzene (15 ml) and dry methylene chloride (0.5 ml). A calcium chloride guard tube was fitted, and the mixture was stirred for 1.5 h. at room temperature, and then for a further 2 h. at 0 °C. Ethyl acetate and 10% hydrochloric acid were then added, and the organic layer was separated, washed with sodium bicarbonate solution, and dried ($MgSO_4$), then concentrated in vacuo. The resulting gum was subjected to preparative tlc (10% acetone - 90% methylene chloride), and the blue fluorescent band gave 2-carbethoxy-3-(2,4,6-trimethoxyphenyl)cyclopent-2-enone (54) (4 mg, 0.01 mmol; 7%) as a crystalline solid, m.p. 110-112 °C (M^+: 320.12598. $C_{17}H_{20}O_6$ requires 320.12599); ν max ($CHCl_3$) 1730, 1700, 1605 cm^{-1}; λ max (MeOH) 206, 224, 336 nm (ϵ 33400, 12400, 7900); δ_H (200 MHz, $CDCl_3$) 1.10 (3H, t, J 7.1 Hz, CH_2CH_3), 2.54 (2H, m, H-5), 3.01 (2H, m, H-4), 3.73 (6H, s, 2x OCH_3), 3.82 (3H, s, OCH_3), 4.15 (2H, q, J 7.2 Hz, OCH_2CH_3), 6.12 (2H, s, 2x Ar-H) ppm; m/z 320 (M^+), 198, 184.

Boron trifluoride catalysed reaction of phenol (50) and bromide (41).

A solution of 3,5-dimethoxyphenol (50) (24 mg, 0.16 mmol) and 3-bromocyclopentenone (41) (25 mg, 0.16 mmol) in boron trifluoride etherate (1 ml) was stirred at 70 °C for 2 h., then at room temperature overnight. The mixture was then poured onto water, and extracted with ethyl acetate. The organic layer was dried ($MgSO_4$), and concentrated in vacuo to a solid, which was purified by preparative tlc (5%

acetone - 95% methylene chloride), and the band at R_f 0.2 gave a white solid, m/z 234 (M^+), 191, which was dissolved in a mixture of pyridine (1 ml) and acetic anhydride (1 ml), and allowed to stand at room temperature overnight. Concentration of the solution in vacuo gave a mixture of 3-(2-acetyloxy-4,6-dimethoxyphenyl)cyclopent-2-enone (55) and 3-(4-acetyloxy-2,6-dimethoxyphenyl)cyclopent-2-enone (56) (19 mg, 0.07 mmol; 44%) as a white solid. δ_H (200 MHz, $CDCl_3$) 2.18 (3H, s, O_2CCH_3), 2.30 (3H, s, O_2CCH_3), 2.45 (4H, m, 2x $-CH_2-$), 3.01 (4H, m, 2x $-CH_2-$), 3.76 (6H, s, 2x OCH_3), 3.80 (6H, s, 2x OCH_3), 6.19 (1H, t, J 1.4 Hz, HC=C), 6.25 (1H, d, J 1.9 Hz, Ar-H), 6.30 (1H, t, J 1.3 Hz, HC=C), 6.35 (2H, s, 2x Ar-H), 6.39 (1H, d, J 1.9 Hz, Ar-H) ppm.

3-(2,4,6-Trimethoxyphenyl)cyclopent-2-enone (57).

1,3,5-trimethoxybenzene (30 mg, 0.18 mmol) and 3-bromocyclopentenone (41) (19 mg, 0.12 mmol) were dissolved in boron trifluoride etherate (2 ml), and the solution was stirred at 80 °C for 4.5 h., then at room temperature overnight. The red solution was then poured onto water, and extracted into ethyl acetate. The organic layer was dried ($MgSO_4$), and concentrated in vacuo to a dark oil, which was purified by preparative tlc (5% acetone - 95% methylene chloride), the blue fluorescent band giving 3-(2,4,6-trimethoxyphenyl)cyclopent-2-enone (57) (15 mg, 0.06 mmol; 52%) as a crystalline solid, m.p. 119-123 °C (M^+: 248.10485. $C_{14}H_{16}O_4$ requires 248.10486); ν max ($CHCl_3$) 1670, 1603 cm^{-1}; λ max (MeOH) 205, 225, 322 nm (ϵ 29800, 13400, 15400); δ_H (200 MHz, $CDCl_3$) 2.42 (2H, m, H-5), 3.07 (2H, m,

H-4), 3.78 (6H, s, 2x OCH_3), 3.83 (3H, s, OCH_3), 6.14 (2H, s, 2x Ar-H), 6.38 (1H, t, J 1.7 Hz, H-2) ppm.

3-(4-Hydroxy-6-methoxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran-7-yl)cyclopent-2-enone (59).

Phenol (39) (46 mg, 0.22 mmol) and 3-bromocyclopentenone (41) (60 mg, 0.37 mmol) were dissolved in ether (2 ml) and cooled to 0 °C. Boron trifluoride etherate (1 ml) was then added, and the solution was stirred at room temperature for 3 days, the colour changing from red to black over that time. The mixture was then partitioned between water and ethyl acetate, and the organic layer was dried ($MgSO_4$), and concentrated in vacuo, affording a dark solid, which was purified by preparative tlc (10% acetone - 90% methylene chloride). The band with R_f 0.25 gave the phenol (59) (4 mg, 0.01 mmol; 6%), (M^+: 288.09976. $C_{16}H_{16}O_5$ requires 288.09978); ν_{max} ($CHCl_3$) 1665, 1608 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 2.17 (2H, m, H-3'), 2.26 (2H, m, H-5), 3.17 (2H, m, H-4), 3.54 (1H, m, H-3a'), 3.81 (3H, s, OCH_3), 4.04 (2H, m, H-2'), 6.20 (1H, s, Ar-H), 6.38 (1H, d, J 5.8 Hz, H-8a'), 6.57 (1H, t, J 1.8 Hz, H-2) ppm.

3-(4-Acetyloxy-6-methoxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran-7-yl)cyclopent-2-enone (60).

Phenol (59) (2 mg) was treated with pyridine (0.1 ml) and acetic anhydride (0.2 ml), and left to stand at room temperature overnight. The solution was then concentrated in vacuo, and purified by preparative tlc (5% acetone - 95% methylene chloride). The pale fluorescent band gave the acetate (60) (2mg), as an off-white solid, (M^+: 330.11033.

$C_{18}H_{18}O_6$ requires 330.11034); ν max ($CHCl_3$) 1750, 1670, 1602 cm^{-1}; λ max (MeOH) 205, 226, 304 nm (ϵ 14500, 8400, 8900); δ_H (200 MHz, $CDCl_3$) 2.08 (2H, m, H-3'), 2.33 (3H, s, O_2CCH_3), 2.42 (2H, t, J 4.8 Hz, H-5), 3.20 (2H, m, J 4.7 Hz, H-4), 3.67 (1H, m, H-2'), 3.80 (3H, s, OCH_3), 3.91 (1H, m, H-3a'), 4.08 (1H, t, J 8.0 Hz, H-2'), 6.26 (1H, s, Ar-H), 6.37 (1H, d, J 5.8 Hz, H-8a'), 6.70 (1H, t, J 1.0 Hz, H-2) ppm.

3-(4-Acetyloxy-6-methoxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran-5-yl)cyclopent-2-enone (58).

To a solution of aflatoxin D_1 acetate (4) (3.7 mg, 0.011 mmol) in ethyl acetate (15 ml) was added 10% palladium on carbon (4.6 mg), and the mixture was stirred under an atmosphere of hydrogen for 3 h. The mixture was then filtered through Celite, and the filtrates concentrated in vacuo, to give the acetate (58) (3.0 mg, 0.009 mmol; 80%), (M^+: 330.11033 $C_{18}H_{18}O_6$ requires 330.11034); ν max ($CHCl_3$) 1762, 1695, 1671, 1624, 1601 cm^{-1}; λ max (MeOH) 203, 227, 209, 315 nm (ϵ 14400, 12200, 4700, 4800); δ_H (200 MHz, $CDCl_3$) 2.10 (2H, m, H-3'), 2.20 (3H, s, O_2CCH_3), 2.46 (2H, m, H-5), 2.99 (2H, m, H-4), 3.71 (1H, m), 3.79 (3H, s, OCH_3), 3.88 (1H, m), 4.10 (1H, m, H-2'), 6.16 (1H, t, J 1.8 Hz, H-2), 6.36 (1H, d, J 6.0 Hz, H-8a'), 6.37 (1H, s, Ar-H) ppm.

1,1-Bis(2,4-dimethoxy-6-hydroxyphenyl)methane (75).

A solution of 3,5-dimethoxyphenol (50) (3.0 g, 19.5 mmol), dimethoxymethane (7.8 ml, 89 mmol) and p-toluenesulphonic acid (22 mg) in dry methylene chloride (80 ml)

was brought to reflux, under an atmosphere of nitrogen, equipped with a Soxhlet apparatus and thimble, filled with 4 Å molecular sieves. After 12 h. reflux, the solution was concentrated in vacuo to a gummy solid. Crystallistion from methanol gave 1,1-bis(2,4-dimethoxy-6-hydroxyphenyl)methane (75) (1.46 g, 4.6 mmol; 23%) as white crystals, m.p 163-165 °C, (M^+: 320.12598. $C_{17}H_{20}O_6$ requires 320.12599); ν max (nujol) 3360, 1620, 1590 cm^{-1}; λ max 218, 271 nm (ϵ 36600, 1700); δ_H (200 MHz, $CDCl_3$) 3.74 (8H, s, 2x OCH_3 + Ar-CH_2-Ar), 3.94 (6H, s, 2x OCH_3), 6.10 (2H, d, J 2.4 Hz, 2x Ar-H), 6.17 (2H, d, J 2.4 Hz, 2x Ar-H), 8.03 (2H, s, exch. D_2O, 2x OH) ppm; m/z 320 (M^+), 167.

1,3,6,8-Tetramethoxy[9H]xanthene (78).

1,1-Bis(2,4-dimethoxy-6-hydroxyphenyl)methane (75) (207 mg, 0.65 mmol) was added to concentrated sulphuric acid (5 ml), and the solution was allowed to stand at room temperature for 2 days. The reaction mixture was then poured onto ice (15 g), and the slurry brought to pH 7 by the dropwise addition of 2 M potassium hydroxide solution. Water was added, and the mixture was extracted with ethyl acetate. The organic layer was washed with water, then dried ($MgSO_4$), and concentrated in vacuo, giving an orange oil which was purified by preparative tlc (17% ethyl acetate - 83% (40-60) petroleum ether) to give 1,3,6,8-tetramethoxy[9H]xanthene (78) (52 mg, 0.17 mmol; 27%) as a white solid. (M^+: 302.11541. $C_{17}H_{18}O_5$ requires 302.11542.) νmax (nujol) 1640, 1612, 1560 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 3.61 (2H, s, Ar-CH_2-Ar), 3.78 (6H, s, 2x OCH_3), 3.81 (6H,

s, OCH_3), 6.15 (2H, A of AB, J_{AB} 2.4 Hz, 2x Ar-H), 6.18 (2H, B of AB, J_{BA} 2.3 Hz, 2x Ar-H) ppm; m/z 302 (M^+), 272, 152.

1,3-Dimethoxy-5-(O-methoxymethyl)benzene (72).

A solution of 3,5-dimethoxyphenol (50) (3.00 g, 19.5 mmol) in dry DMF (15 ml) was added dropwise, under an atmosphere of argon, to a stirred suspension of sodium hydride (0.70 g, 29.2 mmol) in dry DMF (15 ml). The mixture was stirred at room temperature, with the evolution of gas, for 2 h., giving a dark suspension. The mixture was then cooled to 0 °C, and a solution of chloromethyl methyl ether (2.2 ml, 2.3 g, 28 mmol) in dry DMF (10 ml) was added dropwise. After the addition was complete, the pale suspension was stirred for a further 3 h. under argon, then overnight open to the atmosphere in the fume-hood. Water (100 ml) was then added to the mixture, and it was extracted with chloroform (3 x 50 ml). The organic layer was washed with sodium bicarbonate solution (7 x 50 ml), brine (100 ml), and finally water (100 ml). The chloroform solution was then dried ($MgSO_4$), and concentrated in vacuo to 1,3-dimethoxy-5-(O-methoxymethyl)benzene (72) (3.66 g, 18.5 mmol; 95%) as an orange oil, which was further purified by bulb-to-bulb distillation (200 °C, 0.7 mmHg) to a pale yellow oil (Found C, 61.0; H, 7.23. $C_{10}H_{14}O_4$ requires C, 60.6; H, 7.12%); νmax (film) 2950, 1600, 1475 cm^{-1}; δ_H (80 MHz, $CDCl_3$) 3.46 (3H, s, OCH_3), 3.75 (6H, s, 2x OCH_3), 5.12 (2H, s, OCH_2O), 6.12 (1H, t, J 2 Hz, Ar-H), 6.21 (2H, d, J 2 Hz, 2x Ar-H) ppm.

2,4-Dimethoxy-6-hydroxybenzaldehyde (81) and 2,6-dimethoxy-4-(methoxymethoxy)benzaldehyde (79).

A 1.6 M solution of n-butyllithium in hexane (0.6 ml, 0.96 mmol) was added via syringe to a stirred solution of methoxymethyl ether (72) (170 mg, 0.86 mmol) and 2,2'-bipyridyl (1 mg) in dry THF (2 ml), under an atmosphere of argon. The solution was heated at reflux for 1 h., then cooled, after which time the red colour was still present, and dry DMF (0.6 ml) was added via syringe. The red colour slowly disappeared, leaving an orange suspension, which was stirred at room temperature for 30 min. The reaction was then quenched by the addition of water, and extracted into ether. The organic layer was washed with saturated ammonium chloride solution, then dried ($MgSO_4$), and concentrated in vacuo to an oil (99 mg). The oil was dissolved in 50% aqueous acetic acid (20 ml) containing one drop of concentrated sulphuric acid. The solution was stirred at room temperature for 20 h., then extracted into ethyl acetate. The organic layer was washed with brine, then dried ($MgSO_4$), and concentrated in vacuo to an oil, which gave two bands on preparative tlc (5% acetone - 95% methylene chloride). The upper band gave 2,4-dimethoxy-6-hydroxybenzaldehyde (81) (40 mg, 0.22 mmol; 26%). δ_H (60 MHz, $CDCl_3$) 3.79 (6H, s, 2x OCH_3), 5.87 (1H, d, J 2 Hz, Ar-H), 5.96 (1H, d, J 2 Hz, Ar-H), 10.13 (1H, s, CHO) ppm.

The lower band gave 2,6-dimethoxy-4-(methoxymethoxy)benzaldehyde (79) (20 mg, 0.09 mmol; 10%) as an oil, (M^+: 226.08411. $C_{11}H_{14}O_5$ requires 226.08412.);

ν max (film) 1679, 1603, 1578, 1467, 1408 cm^{-1}; δ_H (80 MHz, $CDCl_3$) 3.47 (3H, s, $OC\underline{H}_3$), 3.84 (6H, s, 2x $OC\underline{H}_3$), 5.19 (2H, s, $OC\underline{H}_2O$), 6.21 (2H, s, 2x Ar-$\underline{H}$), 10.34 (1H, s, $C\underline{H}O$) ppm.

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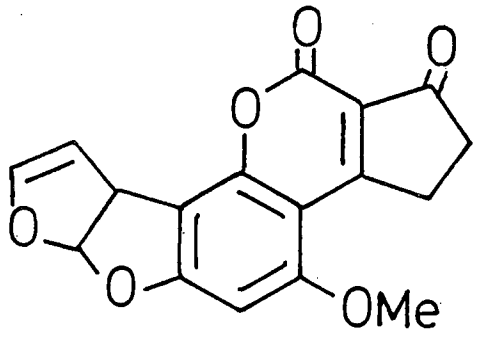
CHAPTER 3

INVESTIGATION OF PROTON NMR SPECTROSCOPY FOR FOLLOWING THE REACTION OF AFLATOXIN B_1 WITH AMMONIUM HYDROXIDE.



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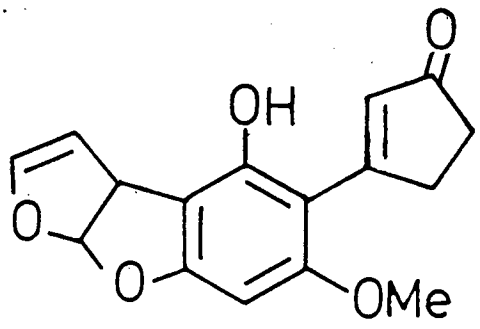

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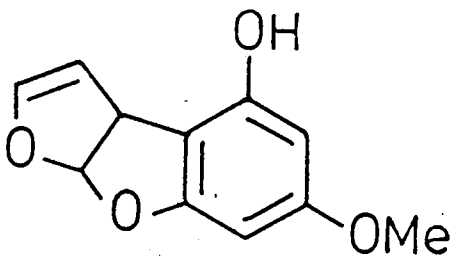

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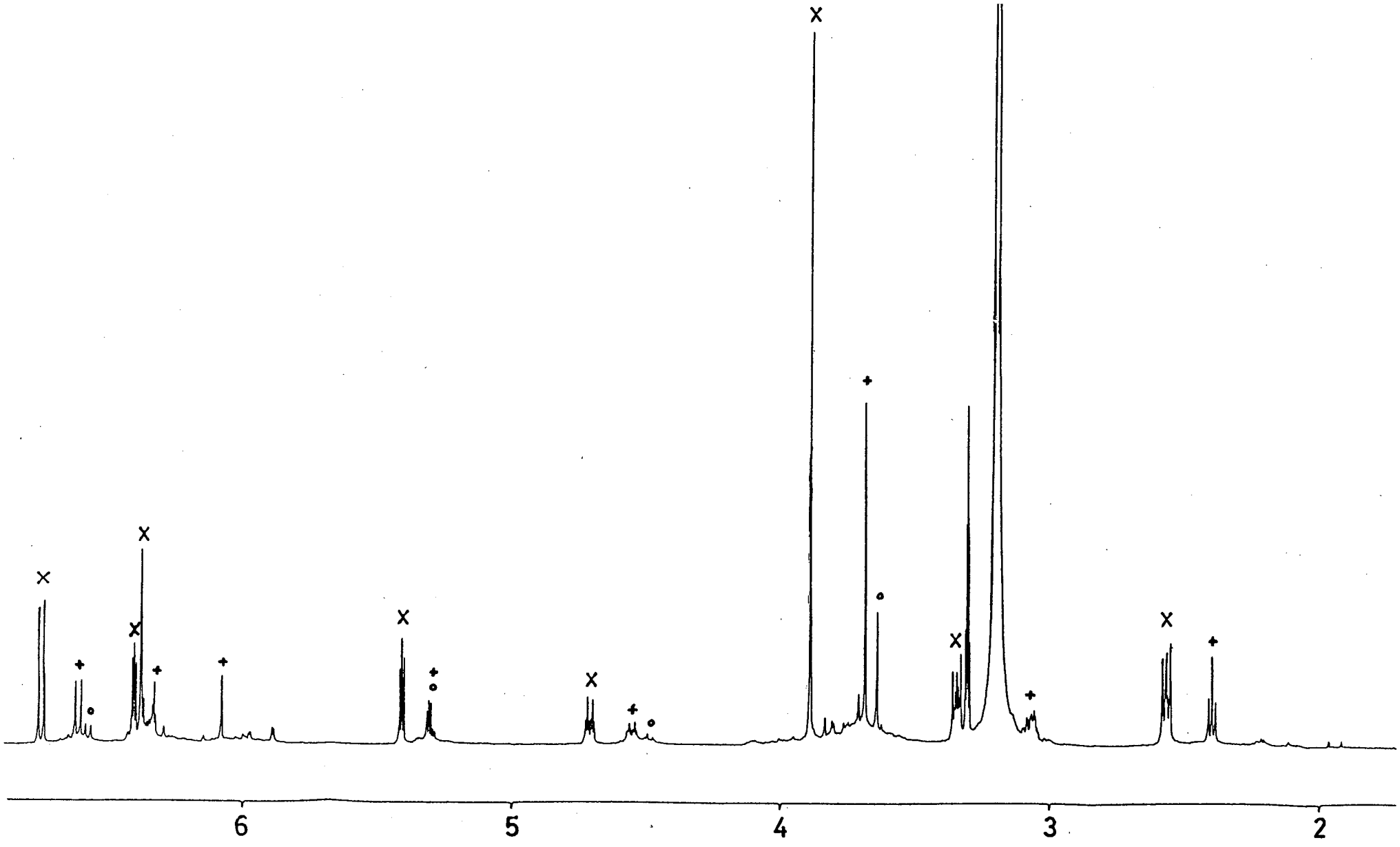


Figure (1) 360 MHz proton nmr spectrum of aflatoxin B_1 ammoniation product, dissolved in $CDCl_3$ - CD_3OD. Selected resonances for aflatoxin B_1 (X), aflatoxin D_1 (+), and MW206 (o) are shown.

3.1 INTRODUCTION.

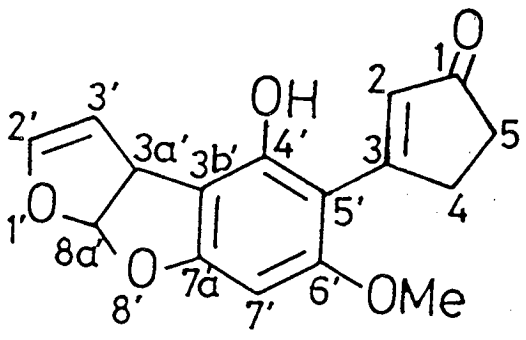
The reported major products of the reaction of aflatoxin B_1 (1) with ammonium hydroxide, i.e aflatoxin D_1[1] (2) and the molecular weight 206 compound[2] (MW206) (3), have been found (Chapter 2) to give rise to characteristic proton nmr spectra. Since no other degradation products could be isolated in significant quantities, it was thought that the mechanism of the ammoniation of aflatoxin B_1 could be studied by using proton nmr to follow the changes in concentration of these compounds with time. In addition, structural evidence for some of the minor products might also be obtained.

3.2 INITIAL STUDIES.

To determine whether or not this approach would be feasible, a lyophilised sample of ammoniated aflatoxin B_1 was dissolved in a mixture of deuterochloroform and deuteromethanol. The proton nmr spectrum of this solution [figure (1)] clearly indicated the presence of aflatoxin B_1, aflatoxin D_1, and MW206. Certain groups of resonances, particularly the doublets due to the acetal protons, were well resolved using high field nmr (360 MHz), and so observation of these resonances in a time course experiment would reveal the changes in concentration of the various constituents in the product mixture bearing the dihydrofurobenzofuran moiety.

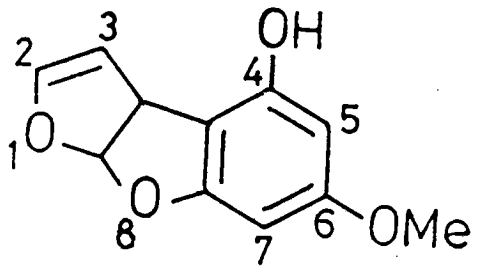
3.3 STUDIES USING DEUTERATED AMMONIUM HYDROXIDE.

The first method to be investigated allowed these changes to be monitored in situ, by observing the proton



Chem. Shift (δ)	Multiplicity	No. H	Assignment
0.424	s	3	OCH_3
1.230	dt (7.1, 2.2 Hz)	1	H-3a'
2.212	t (2.5 Hz)	1	H-3'
2.574	s	1	H-7'
3.180	dd (2.7, 2.1 Hz)	1	H-2'
3.362	s	1	H-2
3.365	d (7.0 Hz)	1	H-8a'

Table (1): 200 MHz proton nmr spectrum of aflatoxin D_1 (2) dissolved in deuterated ammonium hydroxide, with chemical shifts measured relative to the "ND_2H" signal at approx 3.1 ppm.



(3)

Chem. Shift (δ)	Multiplicity	No. H	Assignment
0.383	s	3	OCH_3
1.204	dt (7.1, 2.2 Hz)	1	H-3a
2.186	t (2.6 Hz)	1	H-3
3.160	dd (2.7, 2.1 Hz)	1	H-2
3.330	d (7.0 Hz)	1	H-8a

Table (2): 200 MHz proton nmr spectrum of MW206 (3) dissolved in deuterated ammonium hydroxide, with chemical shifts measured relative to the "ND_2H" signal at approx 3.1 ppm.

nmr spectrum of a sample of aflatoxin B_1, dissolved in deuterated ammonium hydroxide.

In order to be able to distinguish between aflatoxin D_1 (2) and MW206 (3) in the product mixture, reference spectra were run [tables (1) and (2), respectively] for the pure compounds in deuterated ammonium hydroxide.

Unfortunately, possibly due to the unusual solvent, the referencing was not very consistent between spectra. However, all the spectra run in deuterated ammonium hydroxide contained a sharp singlet at ca. 3.1 ppm. This is presumably due to free ND_2H, and when chemical shifts were measured relative to this signal, they appeared consistent to ±0.001 ppm between spectra.

For aflatoxin D_1, the most noticeable differences between this spectrum and that run in a mixture of deuterochloroform and deuteromethanol, were the loss of the methylene resonances by exchange, and the shift to lower frequency of the aromatic proton, presumably due to the formation of the phenolate anion.[3] This aromatic proton also slowly exchanged, although the protons in the spectrum of MW206 exchanged much faster. Apart from the difference in aromatic protons, the spectra of aflatoxin D_1 and MW206 were very similar. Therefore, a mixture of aflatoxin D_1 and MW206 (1.5:1 respectively) was analysed to verify their relative chemical shifts. It could be seen that the resonances for particular protons in aflatoxin D_1 were consistently 0.02 - 0.04 ppm to higher frequency than the corresponding ones in MW206, and that both sets of signals

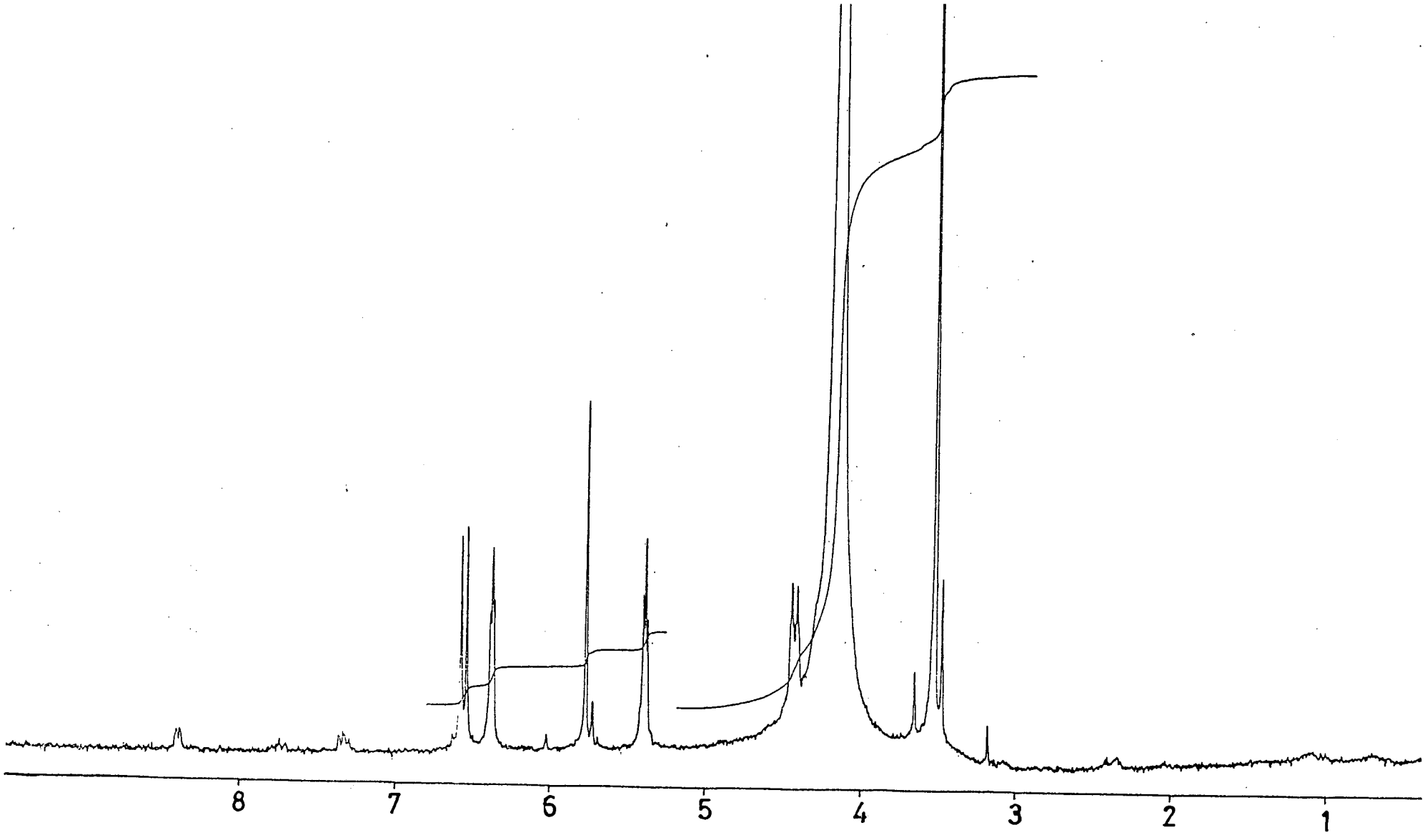


Figure (2) 200 MHz proton nmr spectrum of aflatoxin B_1 dissolved in ND_4OD, after 24 hours at room temperature.

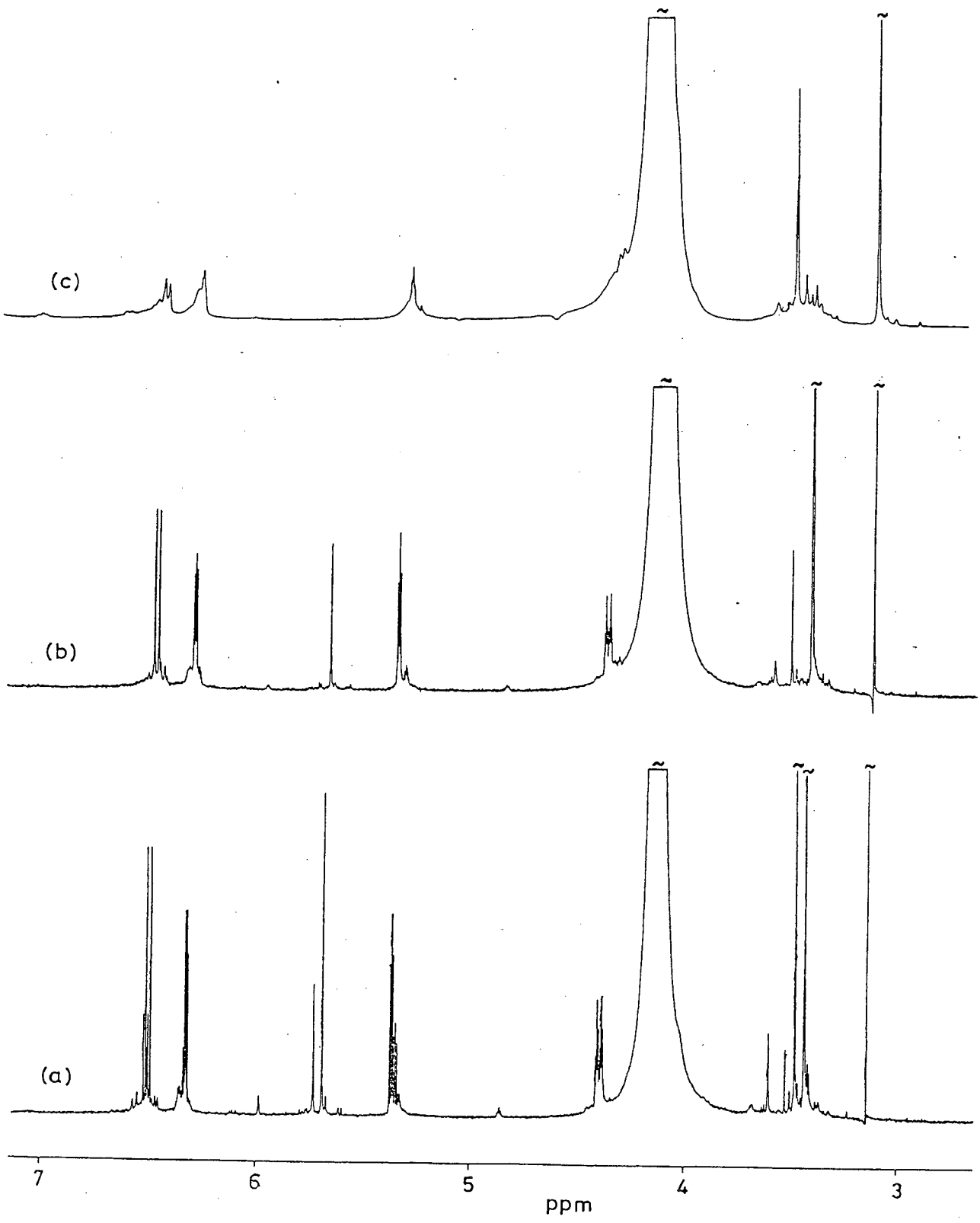
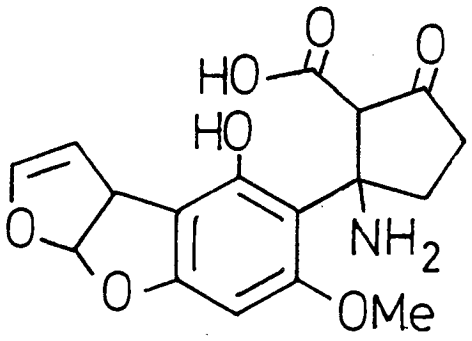


Figure (3) 360 MHz proton nmr spectrum of aflatoxin B_1 dissolved in ND_4OD after (a) 8 days, and (b) 19 days at room temperature, and (c) 19 days at room temperature followed by 10 days at 50°C.



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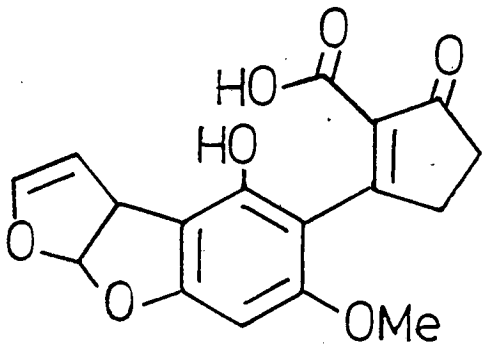

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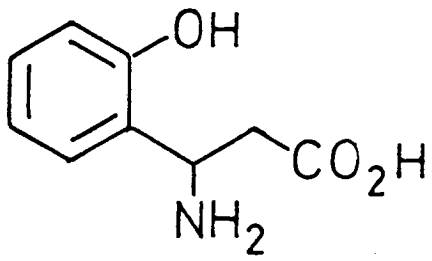

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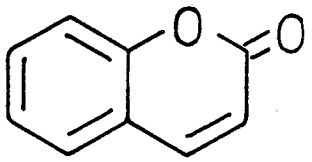


(7)

were present at chemical shift values identical to those obtained in the spectra of the pure compounds, when measured relative to the "ND_2H" signal.

With this information, the results of the nmr studies of aflatoxin B_1, dissolved in ammonium hydroxide could be interpreted.

When aflatoxin B_1 was dissolved in deuterated ammonium hydroxide, the yellow-brown solution gave a proton nmr spectrum [figure (2)] similar to those of aflatoxin D_1 and MW206. In particular, the aromatic proton was at the same chemical shift as that in aflatoxin D_1, suggesting a phenol in a ring-opened form of aflatoxin B_1. The spectrum taken after allowing the solution to stand at room temperature for 8 days [figure (3a)] indicated the presence of new resonances, which differed most from the original ones in the chemical shifts of the aromatic and methoxyl protons, which were both shifted 0.04 ppm to lower frequency. After 19 days [figure (3b)], the original resonances had disappeared completely, being replaced by the new ones seen on day 8. A minor methoxyl resonance was also visible, at the chemical shift consistent with MW206. One possible structure (4) for the major product after 19 days, is that derived by Michael addition of ammonia to the coumaric acid (5). There is a precedent for Michael addition to such systems, since Bergot has reported[4] isolating β-aminohydrocoumaric acid (6) from the reaction of coumarin (7) with ammonium hydroxide.

The sample was then heated at 50 °C for 10 days,

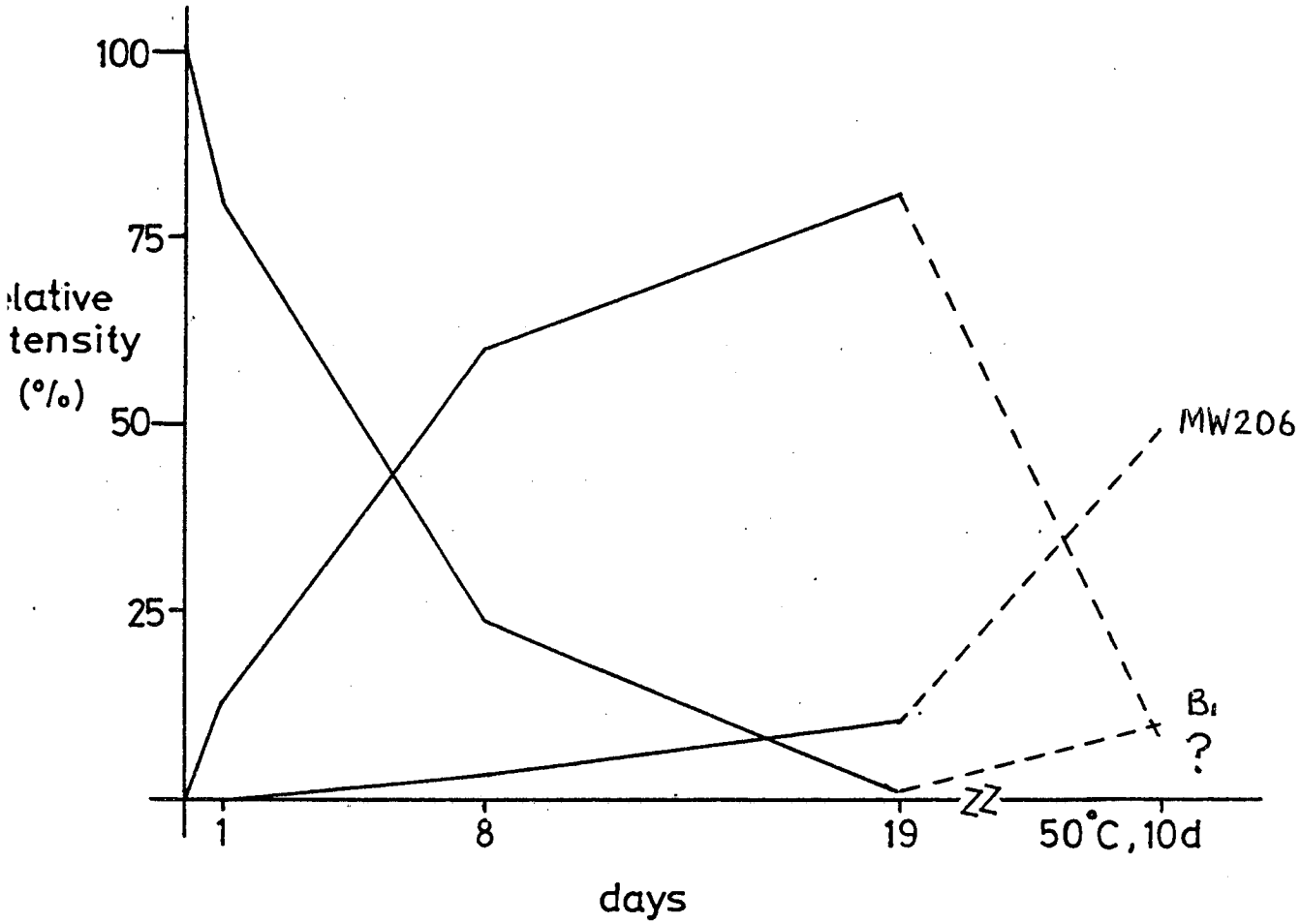


Figure (4) Relative intensities of methoxyl resonances due to products in the reaction of aflatoxin B_1 with ND_4OD at room temperature, and after subsequent heating.

whereupon it turned black. All the groups of resonances in the proton nmr of the product [figure (3c)] showed a degree of line-broadening, and were poorly resolved. The main methoxyl resonance was at a chemical shift corresponding to that in MW206, and the other resonances which could be resolved were all more consistent with the presence of MW206, rather than aflatoxin D_1.

The graph [figure (4)] shows that the initially formed ring-opened product is slowly converted, at room temperature, to another form which still retains the dihydrofurobenzofuran system. Subsequent heating results in the appearance of a single major product, which corresponds closely to MW206. If this product is indeed MW206, then the anomalous production of this compound without the accompanying presence of aflatoxin D_1, must be due to the period of 19 days at room temperature before heating, and could be related to the production of the second ring-opened compound.

Although this method seems to be the most desirable in that no work-up is required in order to observe the nmr spectrum of the reaction mixture, it has been found to suffer the disadvantages that some signals are lost by exchange, and that the quality of the spectrum deteriorates on heating. The use of more standard solvents was therefore reinvestigated.

3.4 NMR STUDIES ON PRODUCTS OF AMMONIATION OF AFLATOXIN B_1, DISSOLVED IN D_5-PYRIDINE.

To follow the ammoniation reaction in this manner, a

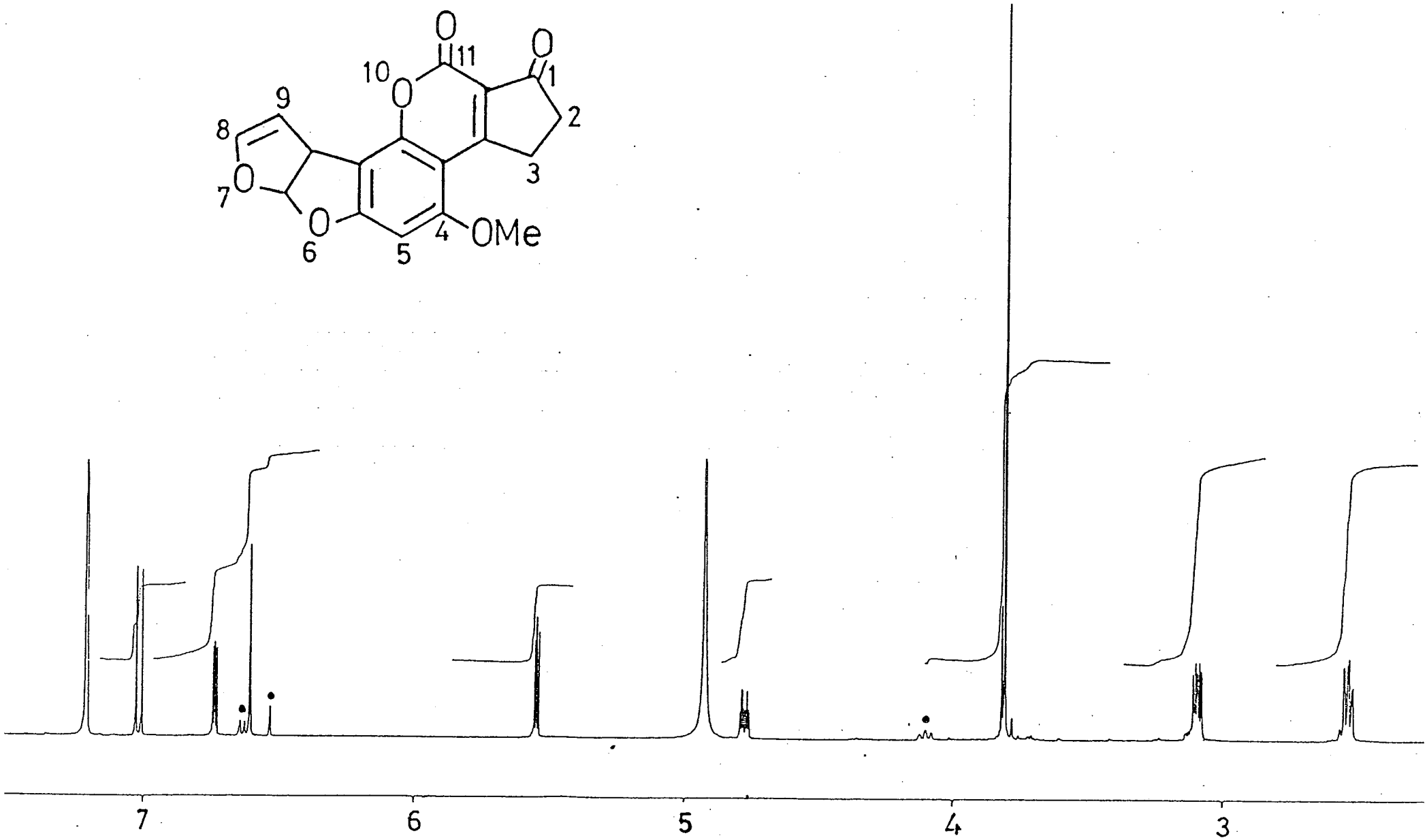
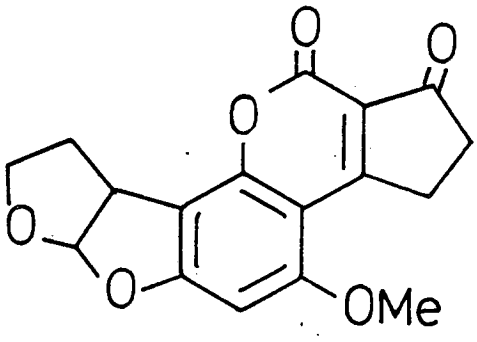


Figure (5) 360 MHz proton nmr spectrum of aflatoxin B_1 dissolved in d_5-pyridine. Selected resonances for aflatoxin B_2 impurity are also shown (•).



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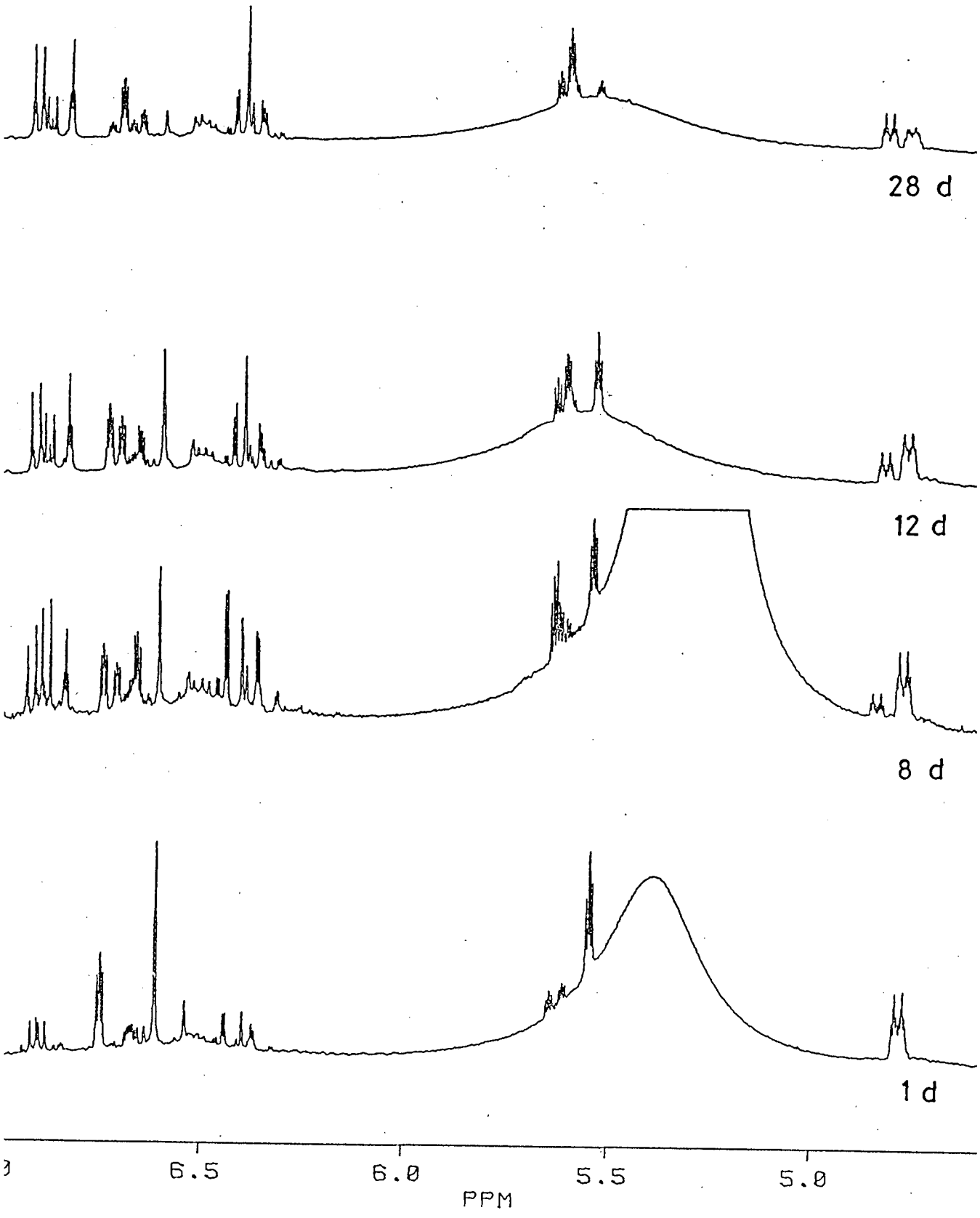


Figure (6) 360 MHz proton nmr spectra of the products of ammoniation of aflatoxin B_1 for 1, 8, 12, and 28 days, run in d_5-pyridine.

solution of aflatoxin B_1 in concentrated ammonium hydroxide was divided equally between a number of flasks, which were all sealed in an identical manner, and placed in an oven at 52 °C. Samples were then removed at intervals, and lyophilised. The samples were dissolved in deuteropyridine and their proton nmr spectra were obtained at 360 MHz. The spectrum of the aflatoxin B_1 sample used was also run [figure (5)], and this indicated that an impurity of ca. 10% aflatoxin B_2 (8) was present. Due to the resolution obtained at high field, this impurity did not interfere, but in fact gave a certain amount of valuable information on the products of ammoniation of aflatoxin B_2 at the same time.

The area of the spectrum which gave the most information was that between 6.3 and 7.1 ppm, containing the acetal, aromatic, and certain olefinic signals. Figure (6) shows how this region changed with time.

By observing the sets of resonances which increase or decrease in concert, between the spectra, it is possible to associate them with a single component. The signals corresponding to aflatoxin B_1 (i.e. the acetal doublet at 7.02 ppm, the olefinic doublet of doublets at 6.74 ppm, and the aromatic singlet at 6.61 ppm) can thus be seen to diminish with time. Similarly, those attributable to aflatoxin B_2 (i.e. the acetal doublet at 6.64, and the aromatic singlet at 6.54 ppm) also decrease.

One set of signals which gradually increases consists of an acetal doublet (J=7 Hz) at 6.92 ppm, an olefinic

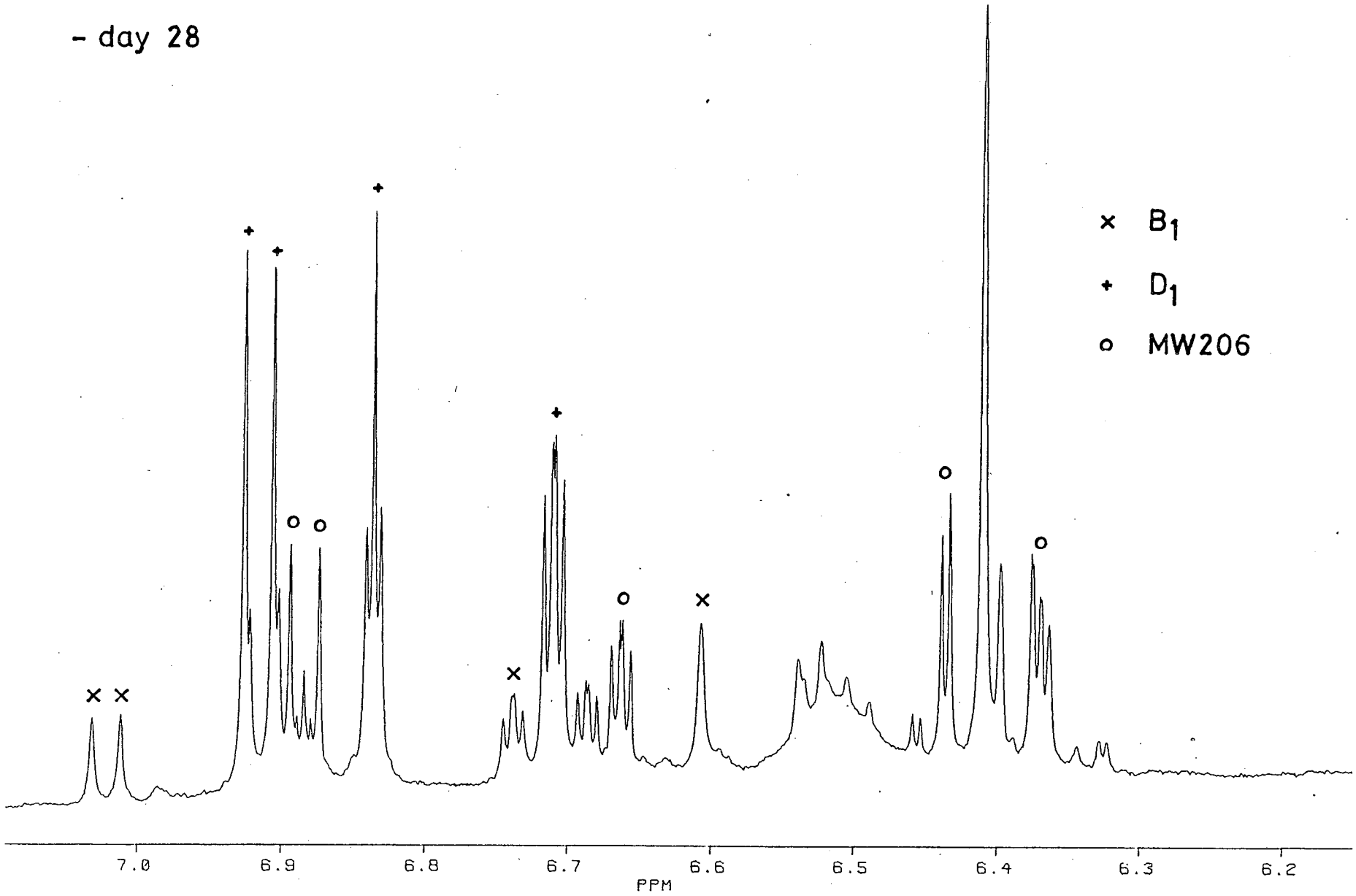


Figure (7) Expansion of the 360 MHz proton nmr spectrum of the product of ammoniation of aflatoxin B_1 for 28 days, run in d_5-pyridine.

triplet (J=1.8 Hz) at 6.83 ppm, an olefinic doublet of doublets at 6.71 ppm, and an aromatic singlet at 6.41 ppm. This set clearly corresponds to aflatoxin D_1.

Another set of signals which increases at a rate different to those due to aflatoxin D_1 can be ascribed to MW206. This consists of an acetal doublet at 6.88 ppm, an olefinic doublet of doublets at 6.66 ppm, and a pair of aromatic doublets (J=2 Hz) at 6.45 amd 6.37 ppm. The lower frequency of the aromatic doublets experiences a broadening effect which the other doublet does not. This is presumably due to a long-range interaction with the methoxyl group.

Interestingly, there is another minor pair of doublets (at 6.47 and 6.32 ppm) which mirror this effect, and increase at a rate similar to those for MW206. It is reasonable to assume therefore, that this is the dihydro-analogue of MW206, derived from aflatoxin B_2. If so, there should also be a corresponding acetal doublet visible for this compound. There are indeed two possible doublets, which appear at 6.49 and 6.53 ppm, and have coupling constants of 5.6 Hz. The remaining doublet should therefore be due to the dihydro- analogue of aflatoxin D_1, which may be called aflatoxin D_2.

If aflatoxin D_2 is present, then an aromatic singlet and an olefinic triplet must also be present in the chemical shift range under investigation. There are two remaining unassigned singlets, at 6.40 and 6.36 ppm, one of which may be due to the aromatic proton of aflatoxin D_2. The olefinic triplet is likely to be the minor one which

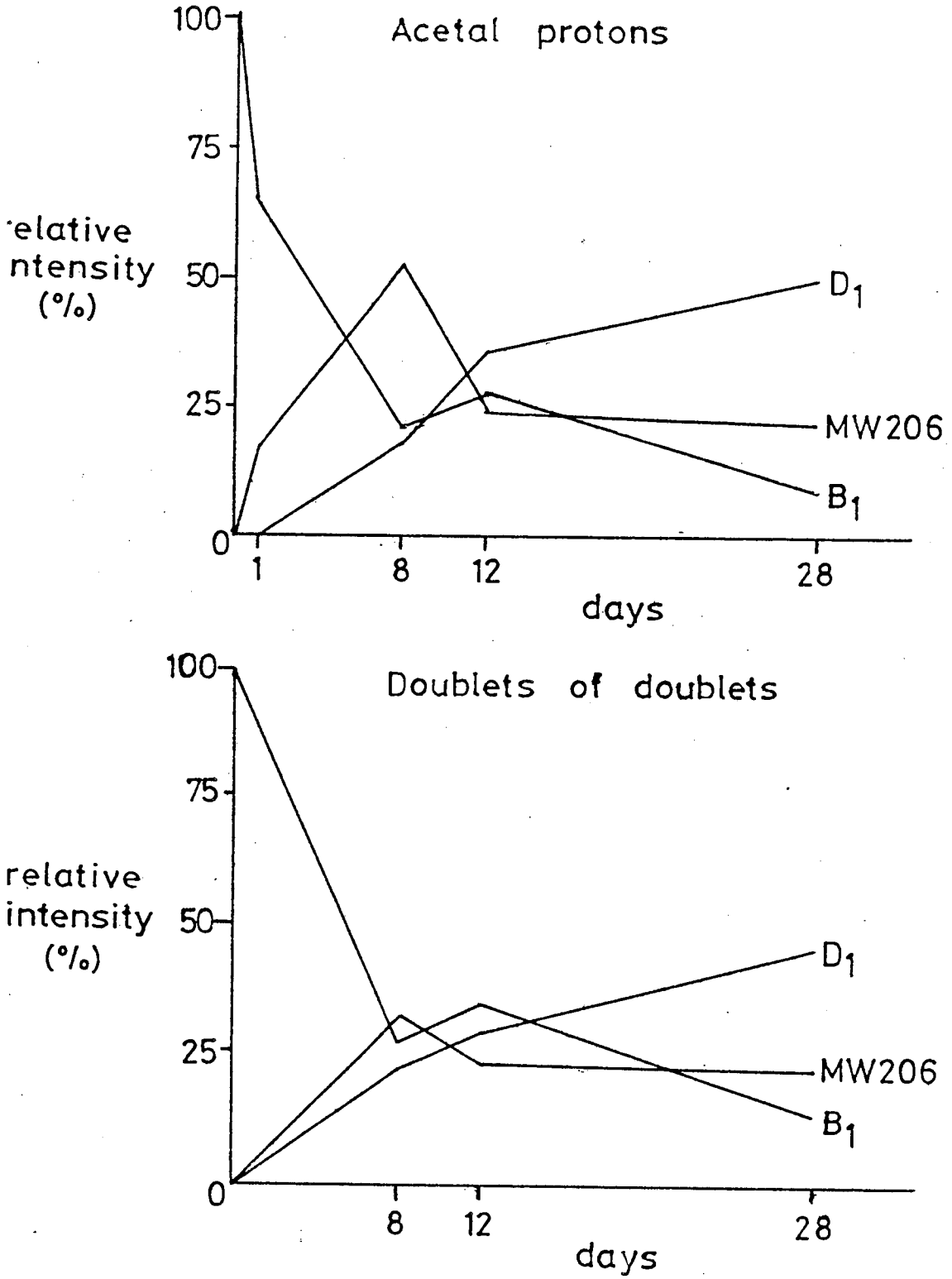


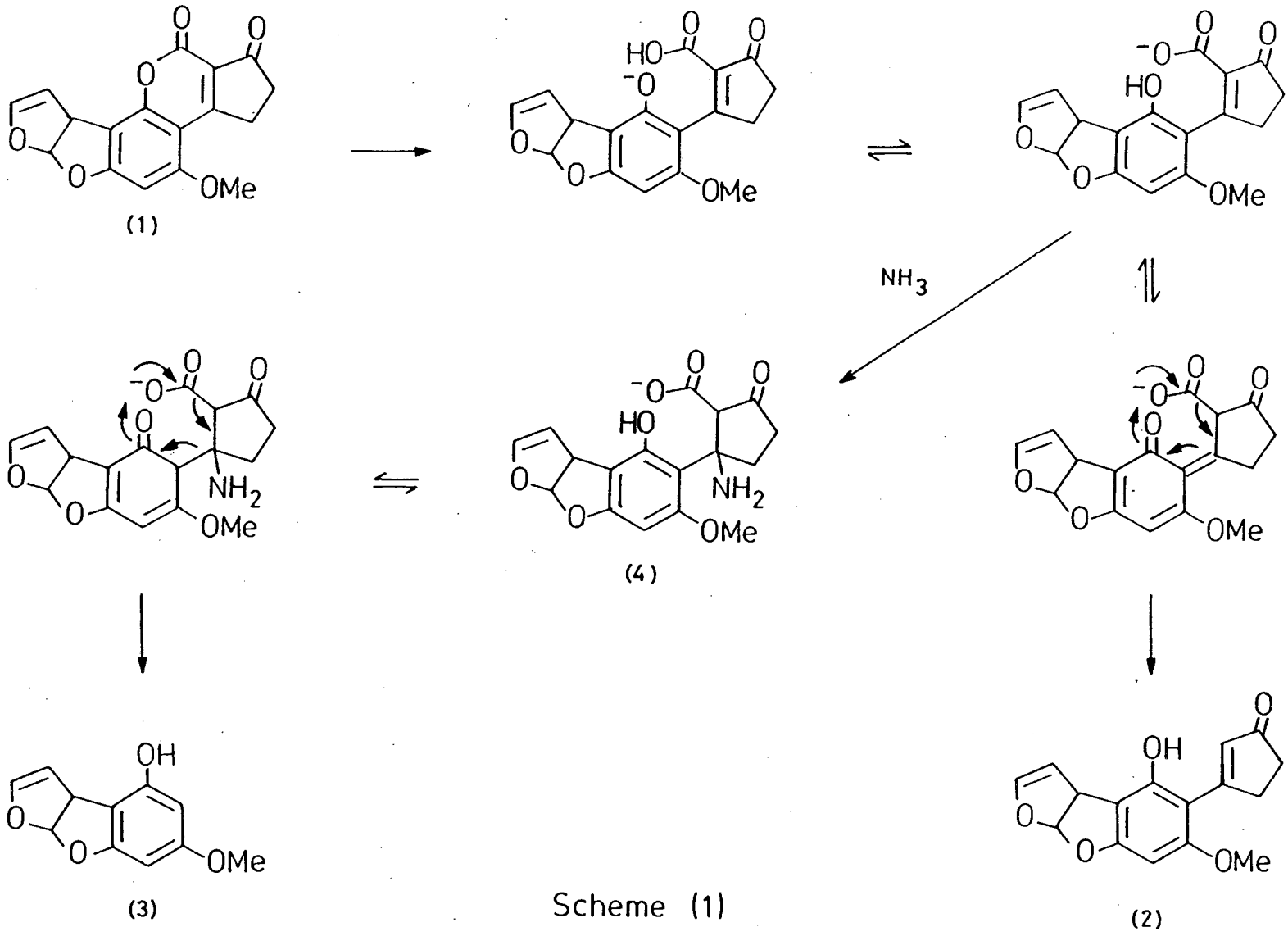
Figure (8) The variation in the relative intensities of resonances corresponding to aflatoxin B_1, aflatoxin D_1, and MW206, with time, during the ammoniation of aflatoxin B_1.

appears at 6.88 ppm [figure (7)], but it may be coincident with the triplet due to aflatoxin D_1.

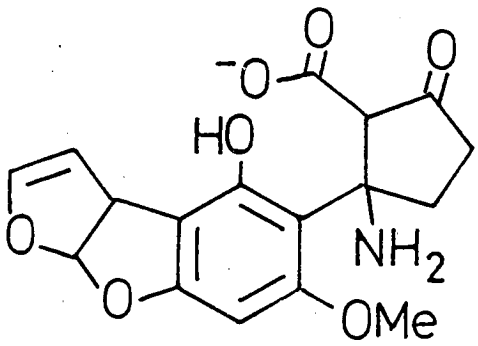
A final minor set of resonances which is visible at day 12 and day 28, consists of an acetal doublet at 6.91 ppm, and an olefinic doublet of doublets at 6.68 ppm. These resonances indicate that this compound contains the dihydrofurobenzofuran moiety, and it is likely that one of the aromatic singlets at 6.40 and 6.36 ppm is also associated with it. The relative intensities of this compound and aflatoxin D_2 are similar, which makes it difficult to determine which compound corresponds with either of the two unassigned aromatic protons, and the minor olefinic triplet. Re-running the experiment on pure aflatoxin B_1, and also on aflatoxin B_2 if possible, would help to resolve this problem.

Having assigned the spectra, the changes in relative concentration of the various components was estimated by observing the change in intensities of the signals corresponding to those components. The graphs [figure (8)] clearly show a decrease in aflatoxin B_1 concentration, and an increase in aflatoxin D_1 and MW206. The trace for MW206 shows that it is not derived from aflatoxin D_1, and it also shows an interesting maximum at day 8. Assuming that this maximum is not merely due to experimental error, it may be due to one or both of the following:

a) The rate of production of MW206 is faster than that of aflatoxin D_1, but it is then further degraded, whereas aflatoxin D_1 is not.



Scheme (1)



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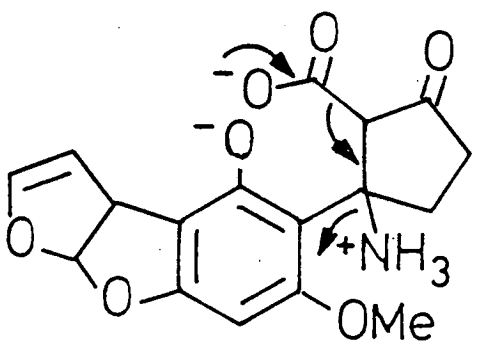

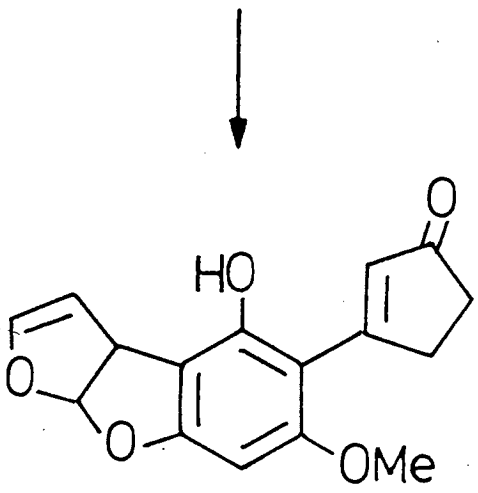

(2)

Scheme (2)

b) The seal on the flasks may not have been perfect, so there may have been a gradual decrease in the concentration of ammonia due to leakage. Aflatoxin D_1 can be considered as arising from a unimolecular decarboxylation of a ring-opened form of aflatoxin B_1, but in order to write a mechanism for the formation of MW206, it is necessary to invoke a Michael addition type process, such as that shown in scheme (1). Therefore, the rate of production of MW206 will be faster at the start of the experiment, when the concentration of ammonium hydroxide is at its greatest. However, it may be possible that at least part of the aflatoxin D_1 formed is also derived from the Michael addition product (4) [scheme (2)].

Also, the fact that dihydro- MW206 and aflatoxin D_2 production mirrors that of MW206 and aflatoxin D_1, confirms the assumption that only the coumarin system is involved in the degradation of pure aflatoxins by ammonium hydroxide.

3.5 CONCLUSIONS.

The above experiments have shown that high field nmr analysis of ammoniation mixtures can indeed be used to monitor the course of the reaction, and also to give structural information on the products as they appear. It is likely that with the use of decoupling experiments and more painstaking analysis of the spectra, yet more information could be obtained. However, since this experiment requires a work-up step, a well-planned, standardised procedure should be adopted to minimise the

chance of the production of artefacts. Further work on the effect of temperature on the production of aflatoxin D_1 and MW206 would be desirable, and in particular, the effect on product distribution of a time-delay before commencing heating should be further investigated.

3.6 EXPERIMENTAL.

Analysis of an aflatoxin B_1 (1) ammoniation mixture by proton nmr spectroscopy.

Aflatoxin B_1 (1) (50 mg) was added to concentrated ammonium hydroxide (20 ml) in a round bottomed flask. The flask was stoppered and sealed, then placed in an oven at 41 °C for 25 d. The product was lyophilised, then hot ethyl acetate (20 ml) was added. The mixture was filtered, and the extraction was repeated, then the combined filtrates were concentrated *in vacuo* to a solid (33 mg). This solid was dissolved in a mixture of $CDCl_3$ and CD_3OD, for ^1H nmr spectroscopy [figure (1)].

Proton nmr spectroscopy of aflatoxin B_1 (1), dissolved in deuterated ammonium hydroxide.

Aflatoxin B_1 (1) (7 mg) was dissolved in d_4-ammonium hydroxide solution (33% in D_2O; 1 ml), and a sample (0.5 ml) was removed to an nmr tube. This was analysed by ^1H nmr after 1 day [figure (2)], 8 days [figure (3a)], and 19 days [figure (3b)] at room temperature. A sample (0.5 ml) was then placed in a round bottomed flask, then stoppered and sealed. The flask was heated in an oven at 50 °C for 10 d., then the product mixture was removed to an nmr tube, and its proton nmr spectrum was recorded [figure (3c)].

Time-course study of the ammoniation of aflatoxin B_1 (1), using proton nmr spectroscopy.

A sample of aflatoxin B_1 (1) [53 mg, containing aflatoxin B_2 (8) (*ca.* 10%)] was dissolved in concentrated ammonium hydroxide (s.g. 0.88; 25 ml), and 5 ml aliquots

were placed in five 25 ml round bottomed flasks. The flasks were stoppered and sealed, then placed in an oven at 52 °C. Flasks were removed after 1, 8, 12, 20, and 28 d., and lyophilised. All the samples, except for day 20, dissolved fully in d_5-pyridine, and were analysed by ^1H nmr [figures (6,7)].

3.7 REFERENCES.

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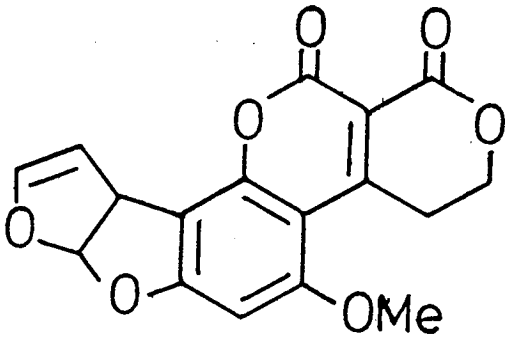
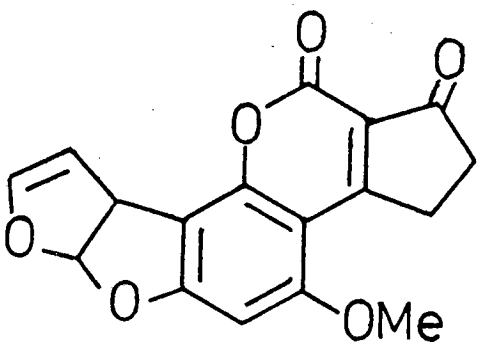
4. B. J. Bergot, W. L. Stanley, and M. S. Masri, J. Agric. Food Chem., 1977, 25, 965.

CHAPTER 4

STUDIES RELATING TO THE AMMONIATION OF AFLATOXIN G_1.



(1)



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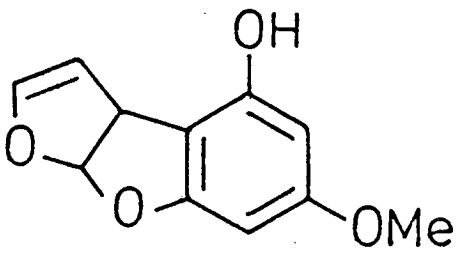


(3)

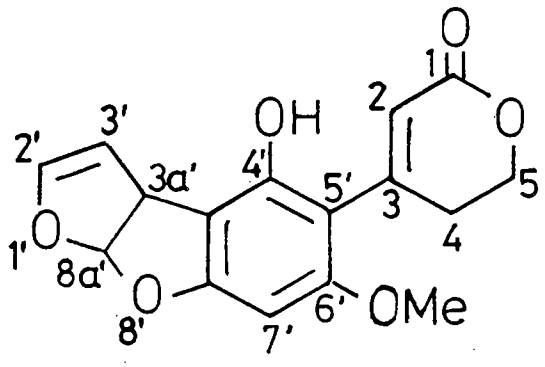
4.1 INTRODUCTION.

Aflatoxin G_1 (1) has been shown, like aflatoxin B_1 (2), to be highly toxic[1] and carcinogenic,[2] but although it is often present in contaminated commodities in amounts comparable with aflatoxin B_1,[3] there has been no published work on the effect of ammoniation on this compound. It was therefore desirable to carry out ammoniations on pure aflatoxin G_1 and to isolate and identify as many of the products as possible.

Previous studies on aflatoxin B_1 have shown that degradation is due to the influence of ammonium hydroxide on the coumarin lactone ring.[4,5] Since aflatoxin G_1 contains two lactone rings, this increases the number of potential degradation products.

4.2 AFLATOXIN G_1 AMMONIATION EXPERIMENTS.

On running an initial experiment by heating 10 mg of aflatoxin G_1 with concentrated ammonium hydroxide, at 56 °C for 14 days, followed by lyophilisation, analytical tlc of the reaction product showed more spots than in the aflatoxin B_1 case. The major product appeared to be a blue fluorescent band which did not migrate far from the base-line. This band was scraped off and the compound eluted, but it was not sufficiently soluble in deuterochloroform or deuteroacetone for nmr analysis. On treating this compound under standard acetylation conditions, aflatoxin G_1 was reformed. This observation suggests that the product was merely a ring-opened form of aflatoxin G_1; the blue fluorescence implying that the coumarin ring was intact.



(4)

Chem. Shift (δ)	Multiplicity	No. H	Assignment
2.74	m	2	H-4
3.75	s	3	OCH_3
4.50	t, J 6 Hz	2	OCH_3
4.61	dt, J 7.3, 1.8 Hz	1	H-3a'
5.38	t, J 2.6 Hz	1	H-3'
6.05	t, J 1.1 Hz	1	H-2
6.14	s	1	H-7'
6.44	dd, J 2.7, 2.1 Hz	1	H-2'
6.69	d, J 7.2 Hz	1	H-8a'

Table (1): 200 MHz proton nmr spectrum of aflatoxin GD_1 (4) in $CDCl_3$.

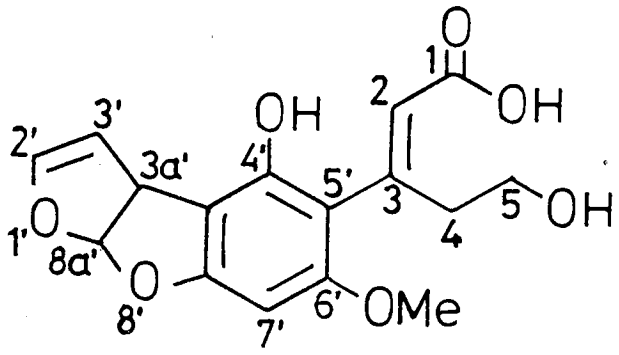
The amount of aflatoxin G_1 that remains in a ring-opened state in the product mixture may be dependent on variables in the work-up procedure, such as lyophilisation conditions, since on repeating the experiment on larger scale, this polar, blue fluorescent band was of minor importance. Analytical tlc of this mixture against standards indicated the presence of aflatoxin G_1 and the molecular weight 206 compound[5] (MW206) (3) amongst the products. MW206 was also isolated by preparative tlc, and its identity was confirmed by nmr.

Two other non-fluorescent bands were visible, which both also gave a positive reaction to spraying with fast blue B[4] (a test for phenols); one of which had an R_f similar to that of aflatoxin G_1, the other having a lower R_f value.

A blue fluorescent band was also visible, with an R_f value less than that of aflatoxin G_1, and giving a negative response to fast blue B.

The upper of the two non-fluorescent bands was removed, giving a substance which was soluble in chloroform. Infra-red analysis showed a carbonyl band at 1712 cm^{-1}, suggesting that at least one lactone functionality was still present. Proton nmr [table (1)] revealed the familiar coupling pattern[1] for the dihydrofurobenzofuran system, and a methoxyl resonance. Also present was an olefinic triplet at 6.05 ppm, with a coupling of 1 Hz. Two methylene resonances were also seen, in positions similar to those in aflatoxin G_1, the one at

(5)



(6)

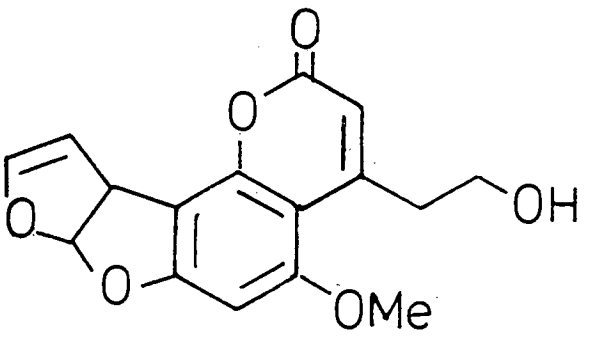
Chem. Shift (δ)	Multiplicity	No. H	Assignment
2.62	m	2	H-4
3.52	m	2	H-5
3.74	s	3	OCH_3
4.61	dt, J 7.2, 2.3 Hz	1	H-3a'
5.39	t, J 2.5 Hz	1	H-3'
5.75	br	1	OH
5.96	t, J 1.3 Hz	1	H-2
6.12	s	1	H-7'
6.43	t, J 2.1 Hz	1	H-2'
6.67	d, J 7.2 Hz	1	H-8a'

Table (2): 200 MHz proton nmr spectrum of 3-(4-hydroxy-6-methoxy-3a,8a-dihydrofuro[2,3b]benzofuran-5-yl)-5-hydroxy-pent-2-en-1-oic acid (6) in $CDCl_3$.

lower frequency apparently coupled to the olefinic triplet. These observations suggested that the compound was the aflatoxin G_1 analogue (4) of aflatoxin D_1 (5). Accurate mass spectrometry also gave a molecular formula of $C_{16}H_{14}O_6$, confirming the above structure, for which the trivial name aflatoxin GD_1 is proposed.

The other non-fluorescent band was also removed, to afford a compound which was more sparingly soluble in chloroform. The infra-red spectrum of this compound showed a carbonyl band at 1660 cm^{-1}, which is too low for a lactone, but consistent with an acid. The proton nmr spectrum of this compound [table (2)] was similar to that of aflatoxin GD_1 (4), except that the higher frequency methylene multiplet was ca. 1 ppm lower in this case, at 3.52 ppm. This chemical shift is more in keeping with protons on a carbon bearing a hydroxyl group than an lactone linkage. It was therefore thought possible that this substance could be the lactone ring-opened form (6) of aflatoxin GD_1, and the lowering of the ultra-violet absorption maximum from 310 nm in aflatoxin GD_1 to 296 nm, is consistent with the disruption of the chromophore on ring-opening. The compound was then submitted for accurate mass spectrometry, giving a molecular formula of $C_{16}H_{16}O_7$, helping to confirm the proposed structural assignment.

Finally, the blue fluorescent band was removed, giving a small quantity (< 1 mg) of substance to analyse. Proton nmr spectroscopy indicated that this was a mixture, containing a "fat-like" impurity. However, resonances for the



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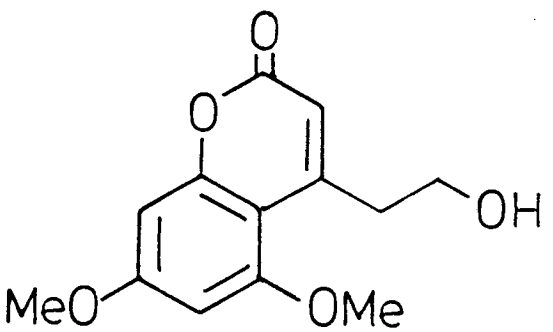

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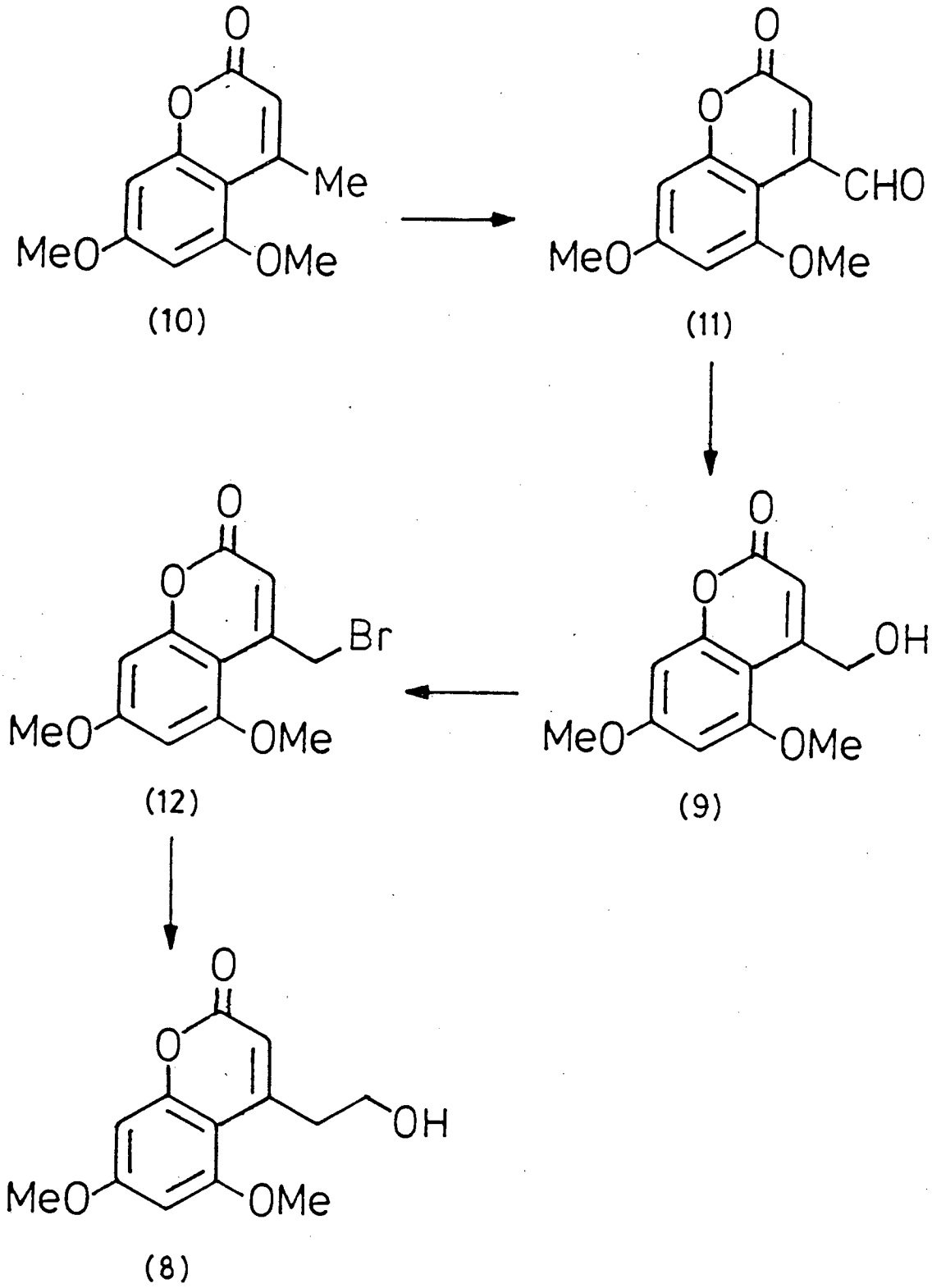

O
O
Me
MeO
OMe
(10)
O
O
CHO
MeO
OMe
(11)
O
O
OH
MeO
OMe
(9)
O
O
Br
MeO
OMe
(12)
O
O
OH
MeO
OMe
(8)

Scheme (1)

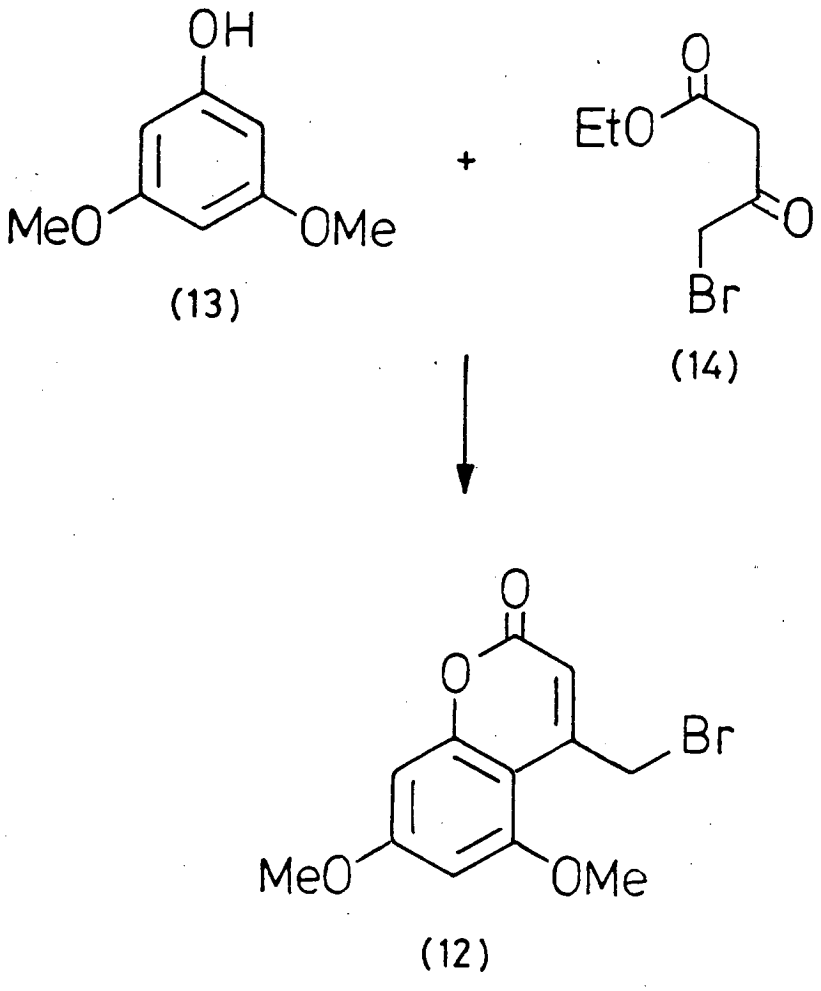
dihydrofurobenzofuran system were clearly visible, as well as an olefinic singlet and a slightly broadened triplet, of coupling 6 Hz, at 3.18 ppm. There was also a multiplet which could not be properly identified, since it was coincident with the methoxyl signal. The compound parasiticol (7) has been reported[6] as a biological degradation product of aflatoxin G_1, having a blue fluorescence under ultra-violet light, and this compound would be expected to exhibit an nmr spectrum similar to that observed for this minor product. Indeed, the methylene, methoxyl, and olefinic resonances in the proton nmr spectrum of the parasiticol model (8) were superimposable with the corresponding signals in the spectrum of the isolated product. Accurate mass spectrometry indicated that this product contained a substance of molecular formula $C_{16}H_{14}O_6$, strongly suggesting that it is indeed parasiticol.

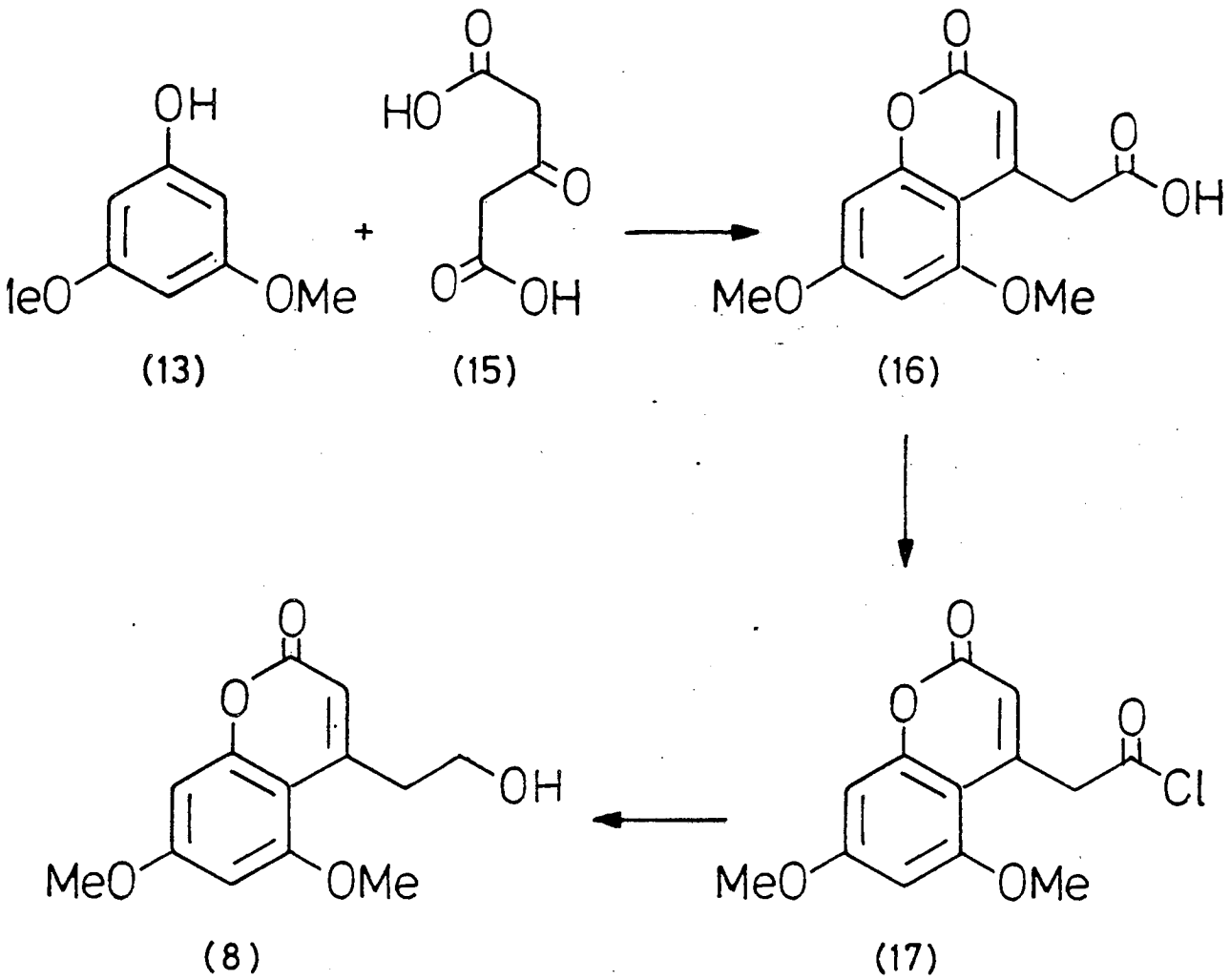
Lack of material and time precluded further studies to purify and rigorously identify this metabolite.

4.3 SYNTHESIS OF MODEL COMPOUNDS.

Synthetic studies were also carried out concurrently with the ammoniation work, with the aim of producing the model (8) of parasiticol. The production of such a model would help identify parasiticol as a possible product in the ammoniation of aflatoxin G_1, as well as providing studies towards a full synthesis.

The intial synthetic approach is described in scheme (1). This was successful as far as the hydroxymethyl


OH
MeO
OMe
(13)
+
O
EtO
O
Br
(14)
O
O
Br
MeO
OMe
(12)


OH
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(8)

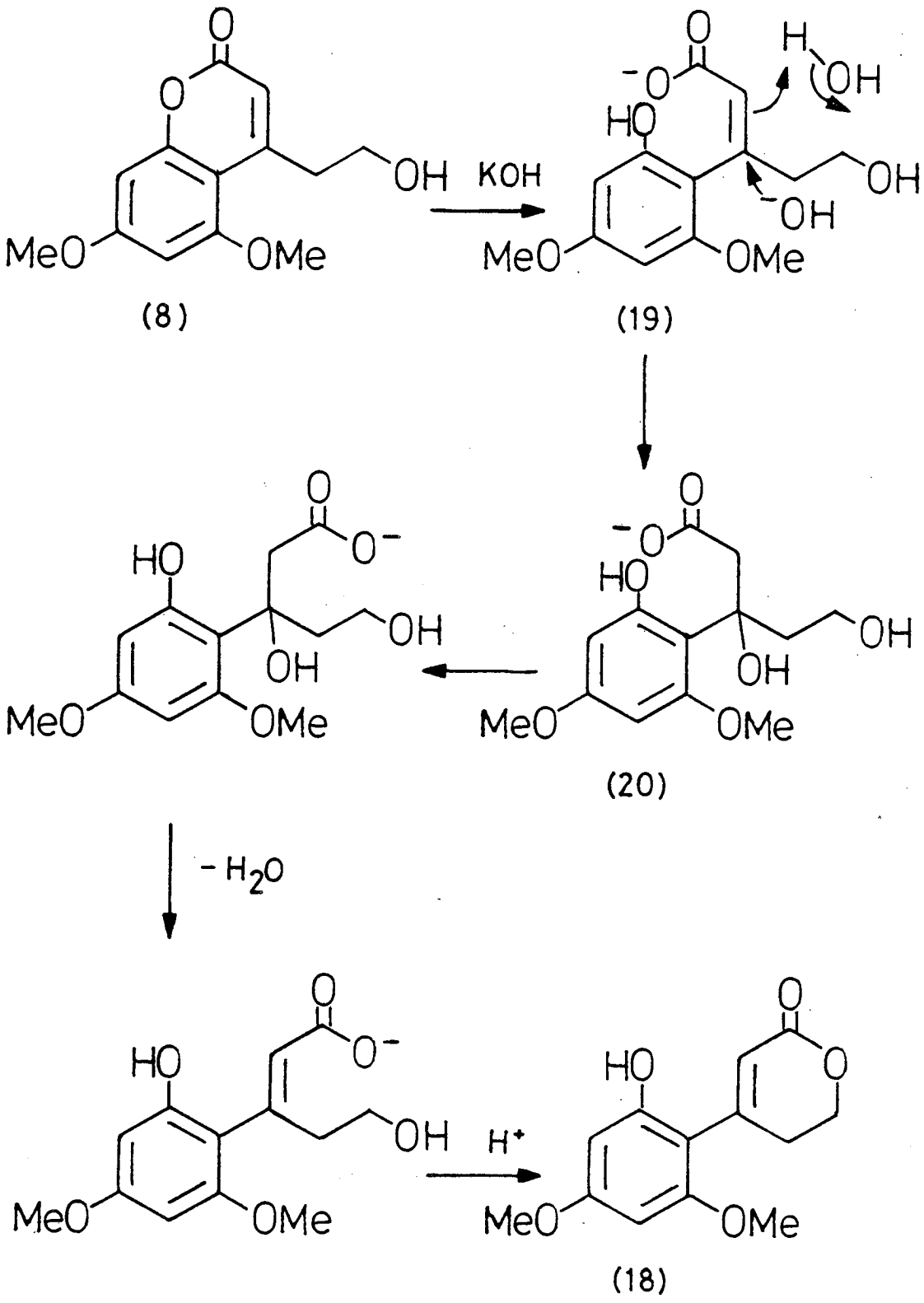
Scheme (2)

coumarin (9), produced from 5,7-dimethoxy-4-methylcoumarin (10) by first oxidising the methyl group to the aldehyde (11) using selenium dioxide in refluxing xylene, then reducing the aldehyde group to an alcohol with sodium borohydride. The required phosphorus tribromide bromination step could not be effected due to the low solubility of the hydroxymethyl coumarin (9) in solvents suitable for the reaction.

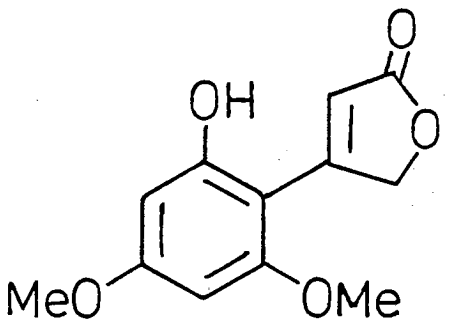
The bromide (12) was synthesised by boron trifluoride etherate catalysed von Pechmann condensation of 3,5-dimethoxyphenol (13) and ethyl 4-bromoacetoacetate (14). However, it failed to react in the Reformatsky reaction with formaldehyde, required to give the desired model (8).

A successful synthesis of the parasiticol model was achieved, and is described in scheme (2). The von Pechmann condensation of 1,3-acetonedicarboxylic acid (15) onto 3,5-dimethoxyphenol, catalysed by boron trifluoride etherate, afforded the coumarin-4-acetic acid (16) in 59% yield. The acid chloride (17), prepared by treatment of the acid with oxalyl chloride, was not isolated, but reacted immediately with sodium borohydride in dry THF to give the desired hydroxyethyl coumarin (8) in 48% yield from the acid.

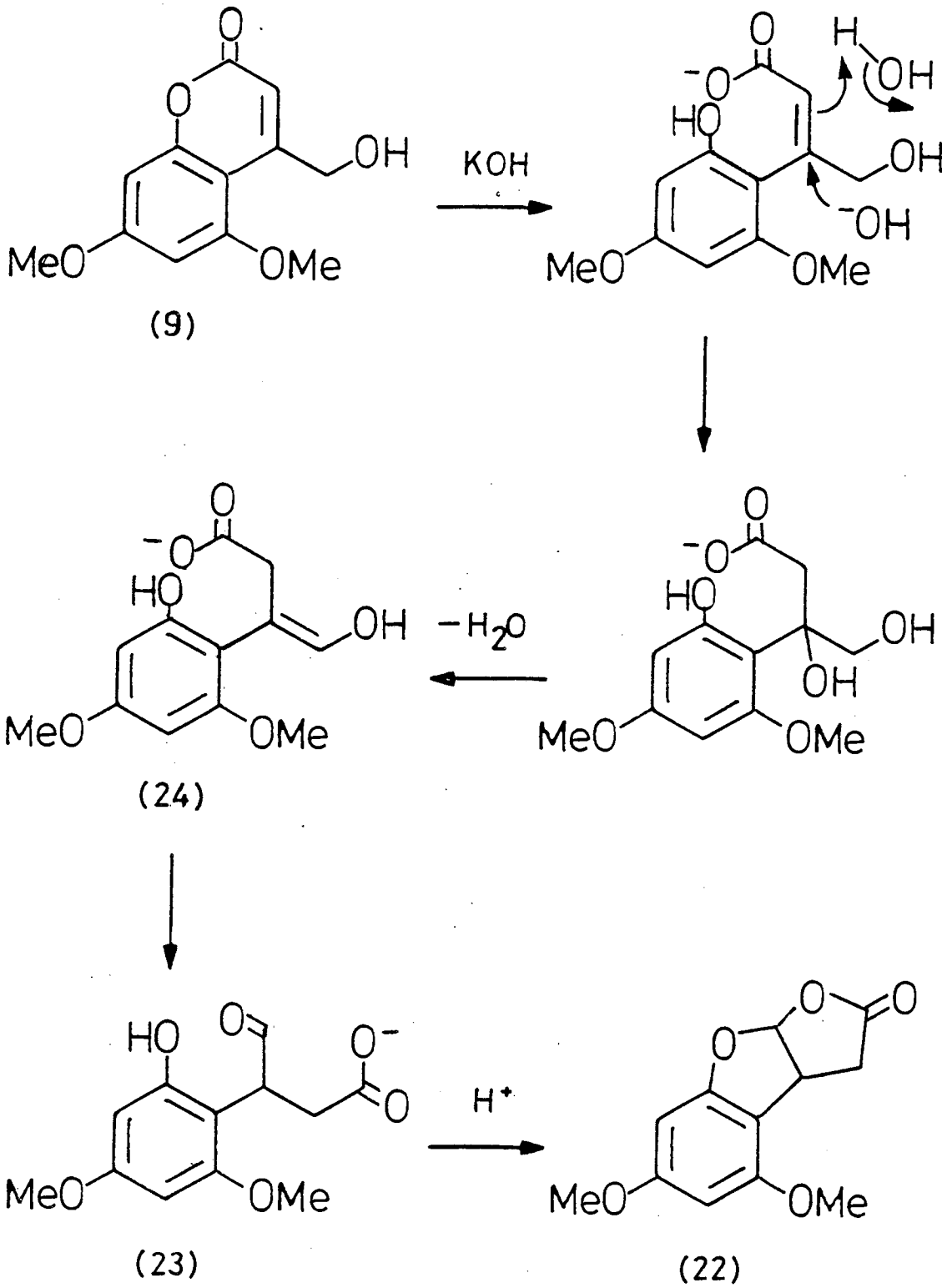
The proton nmr of the parasiticol model (8) bore a very close similarity to that of the compound supposed to be parasiticol, isolated from the ammoniation of aflatoxin G_1, in the chemical shifts of the methylene groups, the methoxyl groups, and the olefinic signal, which was a triplet of coupling 0.5 Hz. The infra-red and ultra-violet



Scheme (3)



(21)



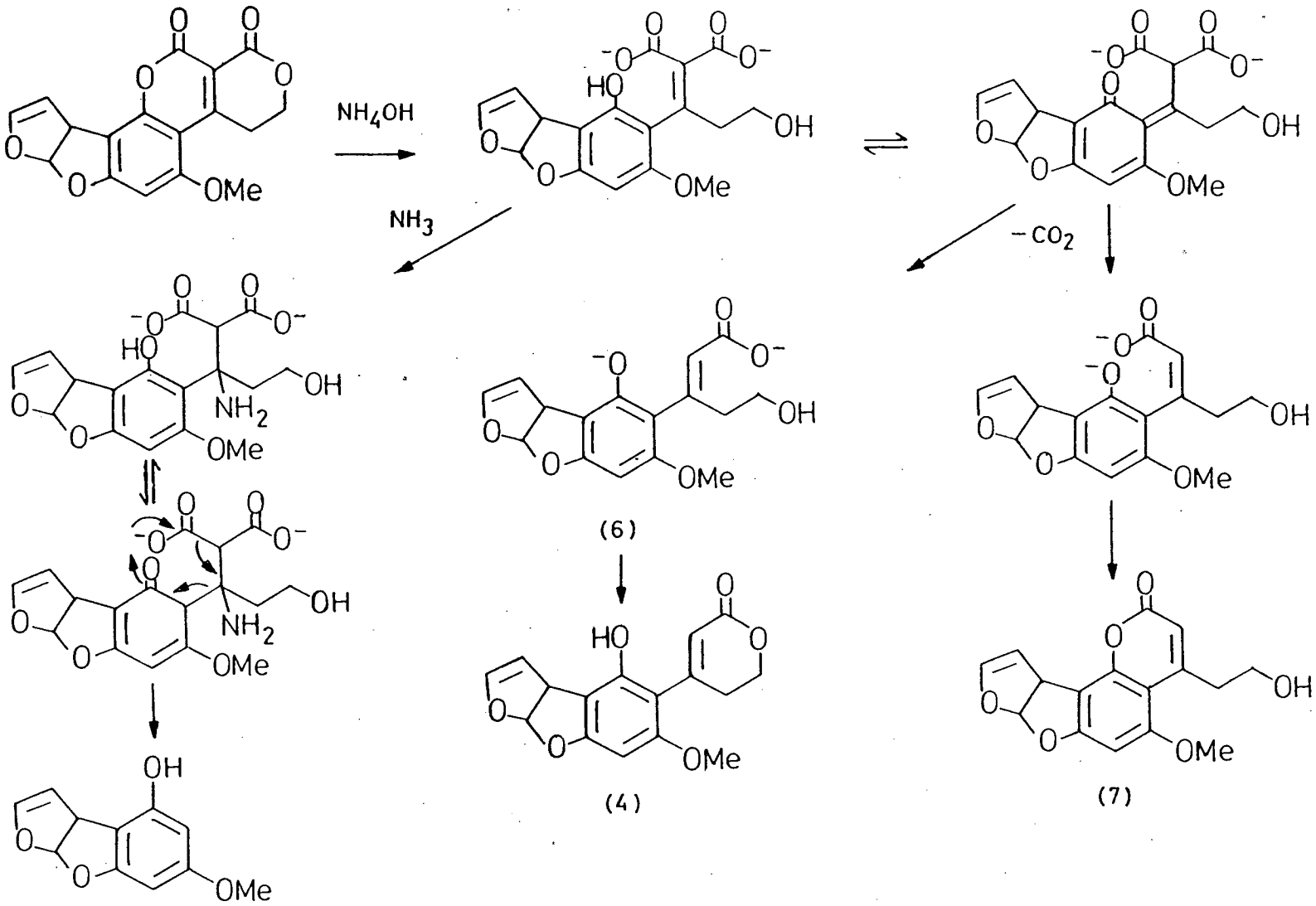
Scheme (4)

spectra were also similar to those quoted[6] for parasiticol.

There also appeared to be an opportunity to produce the corresponding aflatoxin GD_1 model (18) by a base-catalysed isomerisation process [scheme (3)]. This assumes that a ring-opened form (19) will be present in alkaline solution, undergoing base-catalysed addition of water to (20), which allows bond rotation, followed by dehydration, eventually giving the isomer (18).

Before any of the parasiticol model was available for this reaction, the hydroxymethyl coumarin (9) was treated with reluxing methanolic potassium hydroxide solution, to test the feasibility of the above isomerisation. In this case, the lactone (21) was expected, but instead, the tricyclic lactone (22) was isolated in 14% yield from the reaction mixture. This observation may be explained by an addition - elimination process, giving the alkene (24) which tautomerises to the aldehyde (23), then ring closes to give the observed product (22), as shown in scheme (4).

Refluxing the fluorescent parasiticol model (8) with methanolic potassium hydroxide for 22 hours, resulted in a 10% conversion to the expected non-fluorescent lactone (18). The IR and UV spectra of this compound were very similar to those of aflatoxin GD_1, and accurate mass spectrometry showed that it was indeed an isomer of the hydroxyethyl coumarin (8). The proton nmr spectrum revealed two methylene multiplets: a triplet of doublets at 2.74 ppm, with couplings of 6.0 and 1.5 Hz, and a triplet of coupling 6.0 Hz at 4.49 ppm. The corresponding resonances



Scheme (5)

in aflatoxin GD_1 (4) occured at 2.74 and 4.50 ppm. Also of interest was the olefinic triplet, of coupling 1.5 Hz, at 6.07 ppm, since the corresponding triplet in aflatoxin GD_1 was seen at 6.05 ppm.

4.4 CONCLUSIONS.

Ammoniation of aflatoxin G_1 gives rise to a greater number of degradation products than does aflatoxin B_1, which are related to transformations at the lactone rings. Synthesis of model compounds greatly assisted in positively identifying these products, and the fact that model (8) could be transformed to (18) by treatment under basic conditions implies that parasiticol (7) and aflatoxin GD_1 (4) might be similarly interconvertible. On the basis of these facts, the mechanism for the degradation, shown in scheme (5), seems reasonable.

In conclusion, it appears that ammoniation should be at least as effective in degrading aflatoxin G_1 in foodstuffs as it is in degrading aflatoxin B_1.

4.5 EXPERIMENTAL.

Ammoniation of Aflatoxin G_1 (1).

(a) Aflatoxin G_1 (1) (10 mg; 0.03 mmol) was placed in a round bottomed flask. Concentrated ammonium hydroxide solution (s.g. 0.88; 10 ml) was added, and the flask was stoppered and sealed, then placed in oven at 56 °C for 14 d. The product was then lyophilised, and the resulting dark solid was subjected to preparative tlc (10% methanol - 90% methylene chloride). The blue fluorescent band at R_f 0.05 was removed, giving a solid (4 mg). Pyridine (0.5 ml) and acetic anhydride (0.5 ml) were added to the solid, and the solution was allowed to stand at room temperature for 20 min., then was added to crushed ice. The mixture was extracted with ethyl acetate, and the organic layer was dried ($MgSO_4$), and concentrated in vacuo, giving aflatoxin G_1 (1). δ_H (80 MHz; $CDCl_3$) 3.43 (2H, m, H-4), 3.91 (3H, s, OCH_3), 4.40 (2H, m, H-3), 4.75 (1H, dt, J 7, 2 Hz, H-10a), 5.46 (1H, t, J 2 Hz, H-10), 6.43 (2H, m, H-9, and H-6), 6.79 (1H, d, J 7 Hz, H-7a) ppm.

(b) A mixture of aflatoxin G_1 (1) (75 mg, 0.23 mmol) and ammonium hydroxide solution (s.g. 0.88; 5 ml) was sealed in a flask, and placed in an oven at 50 °C for 20 d. The contents of the flask were then lyophilised, and the dark solid was subjected to preparative tlc (4% methanol - 96% chloroform). The plate was divided into six roughly equal zones, which were removed separately, giving a total of 20 mg of material. The residual silica was covered in acetic acid and left overnight, then filtered, and the filtrates

were concentrated in vacuo to a solid. Preparative tlc (5% methanol - 95% chloroform) of this solid gave aflatoxin G_1 (1) (4.6 mg; 6%) at R_f 0.38. The dark band at R_f 0.30 gave 5,6-dihydro-4-(4-hydroxy-6-methoxy-3a,8a-dihydrofuro[2,3-b]benzofuran-5-yl)-2-pyrone (aflatoxin GD_1) (4) (3.3 mg; 4%), (M^+: 302.07903. $C_{16}H_{14}O_6$ requires 302.07904), ν max ($CHCl_3$) 1712, 1620, 1600 cm^{-1}; λ max (MeOH) 206, 310 nm (ϵ 16600, 4280); δ_H (200 MHz; $CDCl_3$) 2.74 (2H, m, $C\underline{H}_2CH_2O$), 3.75 (3H, s, $OC\underline{H}_3$), 4.50 (2H, t, J 6.1 Hz, $CH_2C\underline{H}_2O$), 4.61 (1H, dt, J 7.3, 1.8 Hz, H-3a'), 5.38 (1H, t, J 2.6 Hz, H-3'), 6.05 (1H, t, J 1.1 Hz, HC=C), 6.14 (1H, s, Ar-H), 6.44 (1H, dd, J 2.7, 2.1 Hz, H-2'), 6.69 (1H, d, J 7.2 Hz, H-8a') ppm.

Analytical tlc (5% methanol - 95% chloroform) of the previously removed zones indicated that some contained dark spots below R_f 0.25. These zones were combined and subjected to preparative tlc (5% methanol - 95% chloroform), the non-fluorescent band of R_f 0.25 giving E-3-(4-hydroxy-6-methoxy-3a,8a-dihydrofuro[2,3-b]benzofuran-5-yl)-5-hydroxy-pent-2-en-1-oic acid (ring-opened aflatoxin GD_1) (6) (3.5 mg; 4.5%), (M^+: 320.08959. $C_{16}H_{16}O_7$ requires 320.08960); ν max ($CHCl_3$) 1660, 1625, 1603 cm^{-1}; λ max (MeOH) 208, 296 nm (ϵ 29000, 5400); δ_H (200 MHz; $CDCl_3$) 2.62 (2H, m, $C\underline{H}_2{}_2CH_2OH$), 3.52 (2H, m, $CH_2C\underline{H}_2OH$), 3.74 (3H, s, $OC\underline{H}_3$), 4.61 (1H, dt, J 7.2 Hz, 2.3 Hz, H-3a'), 5.39 (1H, t, J 2.5 Hz, H-3'), 5.75 (1H, br, OH), 5.96 (1H, t, J 1.3 Hz, H-2), 6.12 (1H, s, Ar-H), 6.43 (1H, t, J 2.1 Hz, H-2'), 6.67 (1H, d, J 7.2 Hz, H-8a') ppm.

The residues from this separation were combined with the material from the remaining zones, and the mixture was separated by preparative tlc (5% acetone - 95% methylene chloride). The dark band at R_f 0.43 gave MW206 (3) (2.2 mg; 2%). δ_H (200 MHz; $CDCl_3$) 3.72 (3H, s, $OC\underline{H}_3$), 4.55 (1H, dt, J 6.6, 2.3 Hz, H-3a), 5.32 (1H, t, J 2.6 Hz, H-3), 5.91 (1H, d, J 2.0 Hz, Ar-H), 6.11 (1H, d, J 1.7 Hz, Ar-H), 6.67 (1H, d, J 7.2 Hz, H-8a) ppm.

The blue fluorescent band gave parasiticol (7) (0.9 mg), (M^+: 302.07903. $C_{16}H_{14}O_6$ requires 302.07904.); δ_H (200 MHz; $CDCl_3$) inter alia 3.18 (2H, t, J 6 Hz, $C\underline{H}_2CH_2O$), 3.87 (3H, s, $OC\underline{H}_3$), 4.77 (1H, dt, J 7, 2 Hz, H-10a), 5.47 (1H, t, J 2 Hz, H-10), 6.02 (1H, s, HC=C), 6.40 (1H, s, Ar-H), 6.45 (1H, dd, J 2.7, 2.2 Hz, H-9), 6.76 (1H, d, J 7 Hz, H-7a) ppm.

5,7-Dimethoxy-4-formylcoumarin (11).

A mixture of 5,7-dimethoxy-4-methyl coumarin (10) (2.20 g, 10 mmol) and selenium dioxide (2.22 g, 20 mmol) in xylene (70 ml) was heated to reflux, using a Dean-Stark trap to remove water as it formed. After refluxing for 4 h., the mixture was allowed to cool slightly, then decanted, leaving behind residual selenium and selenium dioxide. The solvent was then removed in vacuo and the residual solid was dissolved in a minimum of warm methylene chloride and filtered to remove more residual selenium. Concentration of the methylene chloride filtrate in vacuo gave 5,7-dimethoxy-4-formylcoumarin (11) (2.36 g, 10 mmol; 100%) as a yellow solid. Recrystallisation of this solid

from chloroform-ether gave pale yellow amorphous crystals, m.p. 195-196 °C, ν max (nujol) 1730, 1695, 1595 cm^{-1}; λ max (MeOH) 218, 251, 261, 329 nm (ϵ 23600, 5900, 5600, 10700); δ_H (80 MHz, $CDCl_3$) 3.86 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 6.28 (1H, s, HC=C), 6.34 (1H, d, J 2 Hz, Ar-H), 6.48 (1H, d, J 2 Hz, Ar-H), 10.46 (1H, s, CHO) ppm.

5,7-Dimethoxy-4-hydroxymethylcoumarin (9).

Sodium borohydride (24 mg, 0.63 mmol) was added to a solution of 5,7-dimethoxy-4-formylcoumarin (11) (100 mg, 0.43 mmol) in THF (20 ml), and the mixture was allowed to stir overnight. The mixture was then acidified by the addition of 10% hydrochloric acid, and the resulting solution was extracted with ethyl acetate. The organic layer was washed with brine, then dried ($MgSO_4$), and concentrated in vacuo to give crude 5,7-dimethoxy-4-hydroxymethylcoumarin (9) (100 mg, 0.43 mmol; 100%) as a white solid. Recrystallisation from methanol gave a white amorphous powder, m.p. 215-220 (decomp.), (M^+: 236.06847. $C_{12}H_{12}O_5$ requires 236.06848); ν max (nujol) 3370, 1676, 1602 cm^{-1}; λ max (MeOH) 215, 246, 256, 320 nm (ϵ 20500, 6700, 5800, 14050); δ_H (80 MHz, d_6-DMSO) 3.85 (3H, s, OCH_3), 3.86 (3H, s, OCH_3), 4.76 (2H, d, J 1.5 Hz, OCH_2-), 6.34 (1H, t, J 1.5 Hz, HC=C), 6.47 (1H, d, J 2 Hz, Ar-H), 6.58 (1H, d, J 2 Hz, Ar-H) ppm.

4-Bromomethyl-5,7-dimethoxycoumarin (12).

Boron trifluoride etherate (1 ml) was added to a mixture of 3,5-dimethoxyphenol (13) (154 mg, 1.0 mmol) and ethyl 4-bromoacetoacetate (14) (230 mg, 1.1 mmol) and

stirred at room temperature, using a calcium chloride guard tube, for 20 h. The mixture was then poured onto water (100 ml) and extracted into ethyl acetate. The organic layer was dried ($MgSO_4$), and concentrated in vacuo to a solid, which was purified by preparative tlc (2% acetone - 98% methylene chloride). The upper band was removed, to give 4-bromomethyl-5,7-dimethoxycoumarin (12) (178 mg, 0.60 mol; 60%), which was recrystallised from methylene chloride-ether to give pale orange crystals, m.p. 139-141.5 °C (lit.[7] m.p. 133-135 °C), νmax (nujol) 1710, 1625, 1600 cm^{-1}; δ_H (80 MHz, $CDCl_3$) 3.83 (3H, s, OCH_3), 3.90 (3H, s, OCH_3), 4.67 (2H, d, J 0.7 Hz, $BrCH_2$-), 6.21 (1H, t, J 0.7 Hz, HC=C), 6.32 (1H, d, J 2.4 Hz, Ar-H), 6.44 (1H, d, J 2.4 Hz, Ar-H) ppm; m/z 300, 298 (both M^+).

5,7-Dimethoxycoumarin-4-acetic acid (16).

Boron trifluoride etherate (5 ml) was added to 3,5-dimethoxyphenol (13) (1.54 g, 10 mmol) and 1,3-acetonedicarboxylic acid (15) (1.46 g, 10 mmol) at 0 °C, and the mixture was stirred at room temperature for 24 h., protected by a calcium chloride guard tube. The mixture was then poured onto water, giving a yellow precipitate. The solid was filtered off, then washed with water (3x), chloroform (3x), and methanol (3x), then sucked dry, to give 5,7-dimethoxycoumarin-4-acetic acid (16) (1.57 g, 5.95 mmol; 60%). The product was recrystallised twice from acetic acid, giving pale yellow needles, m.p. 192-196 °C (decomp.), (M^+: 264.06338. $C_{13}H_{12}O_6$ requires 264.06339); νmax (nujol) 1720, 1670, 1605 cm^{-1}; λmax (MeOH) 211, 247,

258, 325 nm (ε 32400, 7700, 6600, 14500); δ_H (80 MHz, d_6-DMSO) 3.80 (6H, s, 2x $OC\underline{H}_3$), 3.86 (2H, s, $C\underline{H}_2CO_2H$), 6.11 (1H, s, $\underline{H}C=C$), 6.50 (1H, d, J 2 Hz, Ar-$\underline{H}$), 6.60 (1H, d, J 2 Hz, Ar-$\underline{H}$) ppm; m/z 264 (M^+), 220.

5,7-Dimethoxy-4-hydroxyethylcoumarin (8).

Oxalyl chloride (1.5 ml) was added to 5,7-dimethoxycoumarin-4-acetic acid (16) (61 mg, 0.23 mmol) and the mixture was stirred under an atmosphere of argon for 5 h., at 30 °C, by which time all the solid had gone into solution. The flask was connected to the oil pump to remove the excess oxalyl chloride and residues, leaving the acid chloride (17) as a brown solid. To this solid was added a suspension of sodium borohydride (315 mg, 8.3 mmol) in dry THF (15 ml), and after the inital vigorous bubbling had subsided, the pale slurry was stirred overnight at room temperature, under argon. The reaction mixture was then quenched with water, then 2 M hydrochloric acid, and the solution was extracted with ethyl acetate. The organic extract was washed with brine, then dried ($MgSO_4$), and concentrated in vacuo to a white solid. The solid was purified by preparative tlc (5% methanol - 95% chloroform), and the upper blue fluorescent band gave 5,7-dimethoxy-4-hydroxyethylcoumarin (8) (28 mg, 0.11 mmol; 48%). This was recrystallised from chloroform-ether to give pale amorphous crystals, m.p. 144-146 °C (Found: C, 62.5; H, 5.70. $C_{13}H_{14}O_5$ requires C, 62.4; H, 5.64%); νmax (nujol) 3390, 1688, 1604 cm^{-1}; λmax (MeOH) 207, 223, 246, 255, 321 nm (ε 41000, 14300, 5700, 5200, 13500); δ_H (80 MHz, $CDCl_3$) 1.93

(1H, br, OH), 3.16 (2H, m, CH_2CH_2OH), 3.82 (3H, s, OCH_3), 3.86 (3H, s, OCH_3), 3.88 (2H, m, CH_2CH_2OH), 6.01 (1H, t, J 0.5 Hz, HC=C), 6.28 (1H, d, J 2.3 Hz, Ar-H), 6.42 (1H, d, J 2.3 Hz, Ar-H) ppm.

4,6-Dimethoxy-2-oxo-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran (22).

A solution of 5,7-dimethoxy-4-hydroxymethylcoumarin (9) (100 mg, 0.42 mmol) in 2 M methanolic potassium hydroxide solution (15 ml) was heated at reflux for 3.5 h. The warm solution was then poured onto a mixture of ice (200 g) and 10% hydrochloric acid (25 ml). The mixture was extracted with ethyl acetate, and the organic layer was washed with brine, then dried ($MgSO_4$), and concentrated in vacuo. The white solid produced was subjected to preparative tlc (5% acetone - 95% methylene chloride), and the band with R_f value 0.80 gave 4,6-dimethoxy-2-oxo-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran (22) (14 mg, 0.06 mmol; 14%) as a white crystalline solid, m.p. 147-152 °C (lit.[8] m.p. 154-156 °C), δ_H (80 MHz, $CDCl_3$) 2.89 (2H, m, J 6 Hz), 3.75 (3H, s, OCH_3), 3.79 (3H, s, OCH_3), 4.11 (1H, q, J 6 Hz, H-3a), 6.08 (1H, A of AB, J_{AB} 2 Hz, Ar-H), 6.12 (1H, B of AB, J_{BA} 2 Hz, Ar-H), 6.42 (1H, d, J 6 Hz, H-8a) ppm; m/z 236 (M^+).

5,6-Dihydro-4-(2,4-dimethoxy-6-hydroxyphenyl)-2-pyrone (18).

A solution of 5,7-dimethoxy-4-hydroxyethylcoumarin (8) (30 mg, 0.12 mmol) in 2 M methanolic potassium hydroxide (5 ml) was heated at reflux for 22 h., then 2 M hydrochloric

acid (10 ml) was added, and the solution was stirred for 15 min. The solution was then extracted with ethyl acetate, and the organic extract was washed with brine, then dried ($MgSO_4$), and concentrated in vacuo to a solid. Preparative tlc (10% acetone - 90% methylene chloride) was applied to the crude product, and the dark band with R_f 0.40 was removed to give 5,6-dihydro-4-(2,4-dimethoxy-6-hydroxyphenyl)-2-pyrone (18) (3 mg, 0.01 mmol; 10%), (M^+: 250.08411. $C_{13}H_{14}O_5$ requires 250.08413); νmax ($CHCl_3$) 1708, 1610 cm^{-1}; λmax (MeOH) 205, 308 nm (ϵ 22000, 5600); δ_H (200 MHz, $CDCl_3$) 2.74 (2H, td, J 6.0, 1.5 Hz, $C\underline{H}_2CH_2O$), 3.77 (3H, s, $OC\underline{H}_3$), 3.79 (3H, s, $OC\underline{H}_3$), 4.49 (2H, t, J 6.0 Hz, $CH_2C\underline{H}_2O$), 5.48 (1H, br, O$\underline{H}$), 6.07 (1H, t, J 1.5 Hz, $\underline{H}C=C$), 6.08 (1H, d, J 2 Hz, Ar-$\underline{H}$), 6.12 (1H, d, J 2 Hz, Ar-$\underline{H}$) ppm.

4.6 REFERENCES.

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APPENDIX: LECTURE COURSES

The following courses were attended during the period from Autumn 1984 to Summer 1987.

Microcomputers and Instrumentation - Dr. A. Rowley

NMR Spectroscopy - Dr. I. Sadler

Medicinal Chemistry - several speakers

Modern Organic Chemistry - several speakers

Mass Spectrometry - Prof. K. R. Jennings

Cell Biology - Dr. Phillips

Medicinal Chemistry - Prof. Sammes

3 Years' attendance at organic research seminars.